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Evaluation of subcutaneous forms in the improvement of pharmacokinetic profile of warfarin

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ABSTRACT

We attempted to prepare a subcutaneous pharmaceutical form of warfarin based on a suspension or poly(ε-caprolactone) microparticles to improve patient adherence. The warfarin suspension had a mean particle size of 20.0 μm and *in vitro* release close to 100% in 72 h. Microparticle size and encapsulation efficiencies ranged from 54.0 to 80.0 μm and 37.0 to 47.0%, respectively. After 72 h, warfarin microparticles exhibited *in vitro* drug release ranging from 62.0 to 80.0%. Warfarin subcutaneous dosage forms were administered to rabbits. Plasma concentration of warfarin was determined and biological activity was measured by prothrombin time monitoring. The observed relative bioavailabilities calculated from plasma concentrations and prothrombin times were 54.2 and 92.1%, and 61.8 and 61.4% for suspension and microparticles, respectively.

1. Introduction

Since the discovery of dicoumarol in 1939 (Griminger, 1987), vitamin K antagonists (VKA) have been the mainstay of the anticoagulation therapy. Drugs of this therapeutic class produce their anticoagulant effect by interfering with vitamin K which is a cofactor for gamma-carboxylation of glutamic acid residues in clotting factor proteins II, VII, IX, X, and the endogenous anticoagulant proteins C, S, and Z (Gage and Lesko, 2008). The use of VKA is limited by several drawbacks such as delayed onset and offset of action, narrow therapeutic index, inter-individual variability in dose response and multiple food and drug interactions (Ansell, 2007). Although several strategies such as frequent international normalized ratio (INR) monitoring or the study of genetic polymorphisms to optimize warfarin dosing (Becquemont, 2008; Gage and Lesko, 2008; Glurich et al., 2010; Li et al., 2008) have been developed to provide a convenient and affordable approach in the long-term outpatient management of thrombotic disorders (Fareed et al., 2008), the improvement of patient adherence to their anticoagulation therapy still remains an important key point for a safe use of VKA. The IN-RANGE study showed that 35% of patients admitted to specialized anticoagulation clinics had clinically significant levels of poor adherence corresponding to 1–2 missed doses a week (Kimmel et al., 2007). This poor adherence contributes to poor control of anticoagulation; leading to sub-therapeutic anticoagulation periods and a greater risk of stroke or recurrent thromboembolism, whereas extra doses increase the risk of over anticoagulation, which is the major risk factor for bleeding complications (Platt et al., 2008). Adherence could be improved by both better education and training of the patient and also by a greater implication of clinicians who could continue to emphasize strict adherence throughout the course of therapy. Alongside these clinical strategies, some authors have attempted to develop drug delivery systems to modify pharmacokinetics profile of warfarin. To the best of our knowledge, two different strategies have been attempted: a hydroxyapatite ceramic system subcutaneously implanted (Tarr et al., 1997) and a conjugate of warfarin with poly(ethylene glycol) (Zacchigna et al., 2004) administered by the oral route. While the ceramic system reported by Tarr et al. showed a sustained release of warfarin during 7–10 days, the polymeric warfarin conjugate described by Zacchigna et al. was able to reduce the burst release effect and the fluctuation of the plasma concentration of warfarin at steady state.

Although the oral route is the most comfortable way of administration, the subcutaneous route allows the administration of sustained release systems able to deliver active substances for a longer period of time. Such a subcutaneous form of vitamin K antagonist would significantly enhance the observance of the patients and render the anticoagulation therapy safe and effective by

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maintaining a constant circulating drug level, decreasing side effects and increasing tolerability.

In this study, we have evaluated a warfarin suspension and warfarin poly(ε-caprolactone) microparticles. Microparticles were manufactured from poly(ε-caprolactone), a biodegradable polymer suitable for sustained drug delivery, by the simple emulsion technique. After characterization of microparticles in terms of size, entrapment efficiency, water content and thermal behaviour, the *in vitro* release of warfarin was examined. Finally, a warfarin suspension and the warfarin microparticles were subcutaneously injected to rabbits and pharmacokinetic parameters were determined from both plasma concentrations and prothrombin time.

2. Materials and methods

2.1. Materials

Warfarin base (pKa 5.0; MW 308.34 Da), Naproxen sodium salt (pKa 4.2; MW 252.20 Da), Poly(ε-caprolactone) (PCL, MW 40,000 Da) and Polyvinyl alcohol (PVA, MW 30–70,000 Da, 88% hydrolyzed) were purchased from Sigma (Steinheim, Germany). Polyvinylpyrrolidone PVP K-17 (Kollidon® 17 PF, MW 9000 Da) was kindly supplied by BASF SE (Ludwigshafen, Germany). All other reagents were of analytical grade and used as supplied.

2.2. Methods

2.2.1. Preparation of warfarin solution and suspension

The solution of warfarin (2.5 mg/mL) was prepared by dissolving warfarin powder in PBS pH 7.4 and 10% v/v ethanol. The suspension of warfarin (5.0 mg/mL) was prepared by suspending warfarin powder in NaCl 0.9% w/v.

2.2.2. Microparticle preparation

The preparation of PCL microparticles was carried out by the simple emulsion technique (Hombreiro Pérez et al., 2000): 50 mg of warfarin base and 250 mg of PCL were first dissolved in 10 mL of methylene chloride by vigorous magnetic stirring for 5 min. The mixture was then poured into 1000 mL of a 0.1% PVA aqueous solution adjusted to pH 1.0 with a solution of HCl 5N. An oil-in-water (o/w) emulsion was formed by extensive stirring (1600 rpm) with a three-bladed propeller for 2 h at room temperature until the organic solvent was removed. Upon solvent extraction/evaporation, the polymer precipitated and the microparticle cores solidified. Microparticles were then collected by filtration, washed extensively with deionized water, and dried at room temperature. Drug-free microparticles were prepared in the same way.

The preparation of PCL/PVP microparticles was carried out by the method described above, adding 50 mg of PVP to the drug organic phase. Drug-free PCL/PVP microparticles were prepared in the same way.

2.2.3. Warfarin encapsulation efficiency

The amount of warfarin encapsulated within the polymeric microparticles was determined after extraction. Briefly, 10 mg of microparticles were accurately weighed and vortex-mixed for 1 min with 0.1 mL of methylene chloride. Then, 10 mL of methanol were added and vortexed for 5 min. After 15 min of centrifugation at 4000 rpm (1440 g), 50 μL of the supernatant (i.e. methanol) were injected in the HPLC system (Shimadzu HPLC 10A VP, Shimadzu, Japan) with UV detection (SPD-10 A VP, Shimadzu, Japan). Separation was performed on a reverse phase C18 column (Uptisphere ODB, 5 μm particle size, 250.0 mm × 4.6 mm, Interchim, France) at 308 nm (SD-10AVP, Shimadzu, Japan). The column temperature was maintained at 35 °C. The mobile phase composed of methanol and ammonium acetate (50 mmol/l; pH 3.74) (67:33 v/v%) was used at a flow rate of 1.2 mL/min. The warfarin calibration curve was linear from 50 to 150 μg/mL (r^2 = 0.999). The drug entrapment efficiency was expressed as the percentage of warfarin entrapped with respect to the theoretical value, whereas the drug loading was presented as the amount of warfarin entrapped per gram of polymer.

2.2.4. Size distribution and morphology analysis

The particle sizes of bulk warfarin powder and all microparticle batches were analysed by laser light diffraction (Mastersizer S, Malvern Instruments, France). The microparticles were dispersed in 2 mL of an aqueous solution of Tween 80 (1%).

The external morphology of microparticles was examined by scanning electron microscopy (Quanta 600 Feg, FEI Company, Lyon, France) using an Everhart–Thornley detector at 5 kV under vacuum conditions.

2.2.5. Water content measurement

The water content of the microparticles was determined by Karl Fisher titration (756 KF Coulometer, Metrohm SA, Switzerland), by adding 10 mg of microparticles 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.

2.2.6. Differential scanning calorimetry analysis

The thermal properties of the warfarin bulk powder and microparticles were determined by differential scanning calorimetry (DSC) (Q10 DSC, TA Instruments, France). The instrument was calibrated using indium as the standard. Samples of 5 mg were heated in sealed aluminium pans from −60 to 200 °C at a scanning rate of 10 °C/min under nitrogen purge, with an empty aluminium pan as reference.

2.2.7. In vitro drug release

The *in vitro* release study was performed in sink conditions. These conditions were established after the determination of the solubility of an excess amount of warfarin in phosphate buffer at room temperature after 3 days of stirring.

Fifty milligrams of warfarin-loaded or unloaded microparticles were suspended in 40 mL of PBS pH 7.4 (Na_2HPO_4 0.64%, KH_2PO_4 0.06%, NaCl 0.59%). The microparticle suspension was gently stirred (200 rpm) at 37 °C in a water bath. At different times (5, 10, 15, 30, 45 min, 1, 2, 4, 6, 24 and 72 h), 1.5 mL of suspension was withdrawn and filtered with a 0.22 μm Millipore® filter. The filtrate was assayed for warfarin and replaced by 1.5 mL of fresh buffer. The amount of warfarin in the release medium was determined by HPLC-UV analytical method (see Section 2.2.3).

2.2.8. In vivo experiments

Experiments were carried out in compliance with the French legislation for animal experiments (authorization no. 54–68).

The *in vivo* study was performed on three groups of six rabbits which were fasted for 12 h before subcutaneous administration of different formulations. The first group of rabbits (3.29 ± 0.35 kg) was treated at 0.6 mg/kg of body weight with the solution of warfarin (2.5 mg/mL). The second group of rabbits (3.83 ± 0.49 kg) was treated at 0.6 mg/kg of body weight with the suspension of warfarin powder (5.0 mg/mL in NaCl 0.9% w/v) prepared just before administration. Microparticles of warfarin were dispersed in an aqueous solution of NaCl 0.9% w/v (50.0 mg/mL) and administered to the last group of rabbits (3.81 ± 0.47 kg) at a dose of 1.2 mg/kg of body weight. Blank microparticles and the vehicles used in warfarin solution and suspension were also administered to check their potential interference with the analytical assay.

Blood samples of 1.5 mL were collected from the marginal ear vein at different times and added to a constant volume of sodium

citrate 0.129 M (0.17 mL). The plasma was separated by centrifugation at 4000 rpm (1440 g) for 10 min and stored at −20 °C before being analysed.

Warfarin concentrations were determined by HPLC. Briefly, 100 μL of HCl 1 M and 2 mL of diethyl oxide were added to 200 μL of plasma in a capped test tube containing 100 μL of distilled water and 40 μL of naproxen sodium as internal standard (0.5 mg/mL). After vortexing for 60 s, the samples were centrifuged for 10 min at 4000 rpm. The organic layer (1.5 mL) was transferred into a glass tube, and evaporated in a water bath at 40 °C. The residue was reconstituted with 100 μL of the mobile phase composed of methanol and ammonium acetate buffer (50 mmol/L; pH 3.74) (67:33 v/v%). Fifty microliters of the solution were injected in the HPLC system (Model Shimadzu HPLC 10A VP, Shimadzu, Japan). Separation was performed as described above. The warfarin calibration curve was linear from 0.05 to 2.50 μg/mL ($r^2 = 0.996$).

The biological activity of warfarin was determined automatically (STA Compact Automate, Diagnostica Stago, France) by monitoring the prothrombin time (PT). Each control and sample (50 μL) was mixed with 100 μL of thromboplastin (Neoplastine® CI 5, Diagnostica Stago, France) with an international sensitivity index (ISI) value of 1.71. Blank plasma from six rabbits was measured to determine the standard PT.

2.2.9. Statistical analysis

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

3. Results and discussion

3.1. Microparticle characterization

Warfarin microparticles prepared by the o/w method were of spherical shape and had a relatively rough surface punctuated by many pores (Fig. 1 a and b).

Warfarin powder exhibited a homogenous particle size (20.0 ± 0.1 μm) whereas unloaded microparticles presented a broad size distribution of size ranging from 54.0 ± 1.0 to 80.0 ± 8.0 μm, for PVP/PCL and PCL respectively (Table 1). The smaller size of the microparticles containing PVP was probably due to a stabilizing effect because PVP increased the viscosity of aqueous external phase (Feczko et al., 2008). All particles loaded with warfarin had similar sizes of around 70 μm with a monodispersed size distribution of loaded microparticles. The stabilizing effect of PVP was not observed in the case of loaded microparticles, suggesting that warfarin interfered with this effect. This size (below 100 μm) is a satisfactory size for *in vivo* injection by the subcutaneous route, avoiding discomfort during and after administration.

Encapsulation efficiencies of warfarin ranged from 37.4 ± 0.9 to 47.4 ± 2.2% (Table 1) corresponding to microparticles with a core loading of 5.70 ± 0.16% and 8.68 ± 0.40%, respectively. Microparticles including PVP in their formulation showed even lower encapsulation efficiency. As described for other molecules, such as ivermectin (Camargo et al., 2010), the presence of hydrophilic PVP inside the structure of microparticles could lead to an increased porosity. Such a porous structure would increase water penetration into the microparticles, facilitating the loss of warfarin into the release medium. These low encapsulation efficiencies were unexpected since warfarin is practically insoluble in the aqueous phase (PVA 0.1%) and a very low pH value was used to limit the loss of the drug by diffusion into the external phase ($pKa_{warfarin} = 5.0$). Such a low encapsulation rate for a poorly water-soluble drug has been described in the literature. Indeed, Wang et al. (2009) used

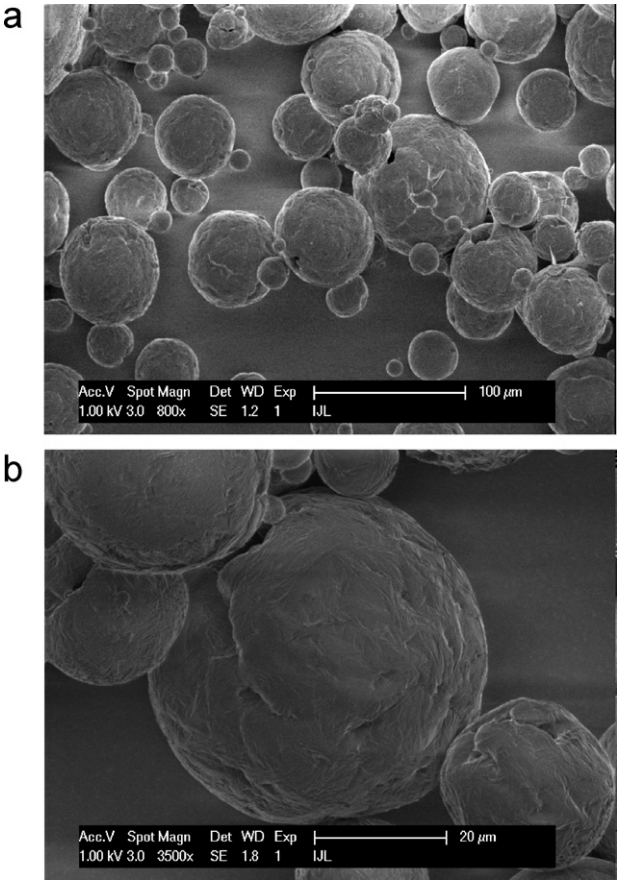

Fig. 1. SEM images of warfarin-loaded microparticles prepared by o/w emulsion.

PCL microparticles to encapsulate p-nitroaniline and found encapsulation efficiencies ranging from 44.0 to 99.0%. It is interesting to notice that the lowest and the highest encapsulation efficiency were found for microparticles with a core loading of 8.20% and 33.02%, respectively. Thus, the further optimizations proposed by Wang et al., such as increasing the surfactant concentration or modifying the PCL/active substance ratios, would certainly improve the encapsulation efficiency of our microparticles. However, since the amount that would have to be administered to the animals based on our encapsulation efficiency has acceptable, we did no further optimization.

The water content of PCL microparticles was 0.59 ± 0.26% w/w whereas it was 1.74 ± 0.01% w/w for PCL/PVP microparticles. Similar results were reported by Raguime et al. (2007) in cellulose acetate and PVP blend membranes; an increase in the water content of the membranes was observed when the PVP concentration was increased. Since the water content represents the fraction of water molecules located in the pores of the membrane, the use of a pore former such as PVP increases the porosity of the membranes leading to an increase of water content. A similar phenomenon would be expected in the case of microparticles, which would lead to more porous and hydrophilic microparticles.

On differential scanning calorimetry analysis, warfarin powder showed a melting temperature of 165.9 °C and the PCL polymer in unloaded microparticles exhibited a glass transition temperature of −61.4 °C and a melting temperature of 60.7 °C (Table 2). In the thermogram of drug-loaded microparticles prepared by the o/w-method, the glass transition and the melting temperatures of PCL were still present whereas the peak corresponding to the melting temperature of warfarin disappeared. This suggests that warfarin is

Table 1
Properties of warfarin microparticles. Data are expressed as mean ± SD (n = 3).

Warfarin microparticles						
Formulations		Mean diameter (μm)	Encapsulation efficiency (%)	Core loading (%)	Water content (%)	Warfarin released at 72 h (μg/mL)
Unloaded	PCL	80.0 ± 8.0	–		–	–
	PCL/PVP 50	54.0 ± 1.0	–		–	–
Loaded	PCL	70.0 ± 6.0	47.4 ± 2.2	8.68 ± 0.40	0.59 ± 0.26	61.7 ± 4.2
	PCL/PVP 50	72.0 ± 1.0	37.4 ± 0.9	5.70 ± 0.16	1.74 ± 0.01	80.0 ± 3.4

Table 2
Thermal properties of warfarin, PCL and microparticles measured by differential scanning calorimetry.

	Compound	T_g (°C)	T_m (°C)
Powder	Warfarin	–	165.9
Unloaded microparticles	PCL	−61.4	60.7
Loaded microparticles	Warfarin	–	–
	PCL	−52.6	58.6

either amorphously dissolved or molecularly dispersed within the polymer matrix of microparticles.

3.2. In vitro drug release

The release profiles of warfarin from microparticles in pH 7.4 phosphate buffer are shown in Fig. 2. The two formulations of microparticles exhibited a similar release profile characterized by an initial burst during which warfarin was rapidly released over half an hour followed by a plateau characterized by a very slow and incomplete release. The burst effect was probably related to the drug entrapped near the surface of the microparticles whereas at the later stage, the drug was released more slowly; in this latter case, the dissolution rate is determined by the diffusion of the drug through the amorphous region of the polymer matrix (Jeong et al., 2003). The amount of warfarin released presented in Table 1 was significantly different between the two different formulations. The amount of active substance released at 72 h was 61.7 ± 4.2% for PCL microparticles, in agreement with previous findings (Gibaud et al., 2004; Homar et al., 2007; Sastre et al., 2004; Wang et al., 2009). The increase in release rate observed with PCL/PVP microparticles (80.0 ± 3.4% at 72 h) did not seem to be related to a difference in specific surface area since the PCL and PCL/PVP had similar diameters (70.0 ± 6.0 and 72.0 ± 1.0 μm, respectively) with a narrow distribution. Thus, it can be inferred that this result is due to the different structures of these two kinds of particles, implicating the pore-forming role of PVP in the increase of warfarin release as described previously (Raguime et al., 2007). In our work, the difference in water content between PCL and PCL/PVP microparticles was close to 3-fold leading to the increase of the volume of diffusion and drug mobility. Finally, the possible role of PVP in the improvement of warfarin solubility in the dissolution medium should be noted. Indeed, some authors have reported an improvement in the dissolution of fairly and poorly soluble drugs as atenolol (Moneghini et al., 1998) acetaminophen (Wen et al., 2005) or celecoxib (Gupta et al., 2005) due to different interactions with PVP such as hydrogen bonds, hydrophobic interaction or van der Waals interaction leading to enhanced stability of the amorphous drug form.

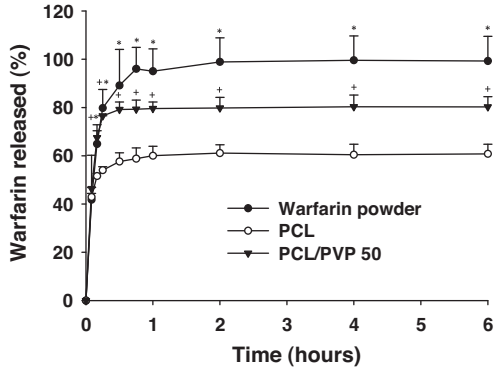

Fig. 2. Release profile of warfarin from microparticles. Experiments were performed in phosphate buffered saline at 37 °C and pH 7.4 under 200 rpm stirring. Data are shown as mean ± S.D. (n = 3). *, +: statistically different compared with PCL microparticles (p < 0.05).

3.3. In vivo experiments

After administration to rabbits of the blank microparticles or the pure vehicles, no interference was observed with the assay methods. The standard prothrombin time (PT) value determined in control plasma was 7.0 ± 0.4 s.

Based on the results of the *in vitro* dissolution profiles, only PCL microparticles, which exhibited the lowest released amount of warfarin, were tested in animals. Both the plasma concentrations and the biological activities of warfarin solution, suspension or microparticles are presented in Fig. 3. The warfarin suspension yielded a constant plasma concentration of about 0.20 μg/mL from 45 min to 34 h. Warfarin microparticles showed a very similar release profile to warfarin solution after a single subcutaneous injection for the first 24 h; that is an initial peak during the first hour followed by a rapid decrease until 24 h. From 24 to 72 h, warfarin microparticles showed a zero order release rate of warfarin leading to a plasma concentration around 0.16 μg/mL, whereas the warfarin concentration decreased to an undetectable level 72 h after the administration of the solution. The maximal plasma concentrations were 1.44 ± 0.20, 0.20 ± 0.03 and 1.17 ± 0.50 μg/mL for the solution, the suspension and the microparticles, respectively (Table 3) and were reached at 0.75, 3 and 1.5 h for warfarin solution, for warfarin suspension and microparticles, respectively. The values of AUC were 7.34 ± 1.67 and 22.50 ± 5.63 h.μg/mL and the values of relative bioavailability were 54.2 ± 18.8 and 92.1 ± 18.6% for the suspension and microparticles, respectively. It is interesting to notice that although warfarin microparticles and warfarin solution showed no statistically difference in relative bioavailability, peak concentration, time to peak concentration or AUC between 0 and 24 h (14.38 ± 5.16 h μg/mL and 10.48 ± 0.70 h μg/mL, respectively), they did give a statistically different plasma concentration profile and AUC between 24 and 72 h (8.11 ± 0.48 h μg/mL and 1.68 ± 0.48 h μg/mL, respectively) (Table 3). Thus, while the total amount of drug available is the same, the drug release process is different, with microparticles providing a sustained release of warfarin from 24 to 72 h. It could be hypothesized that warfarin near the surface of the microparticles could be rapidly bioavailable compared with warfarin inside the particle matrix: this deeper localization allows migration of warfarin to the surface. However, it is unclear why the *in vitro* and *in vivo* release results for warfarin from suspension and microparticles are not in agreement. The poor bioavailability of the warfarin suspension obtained in our

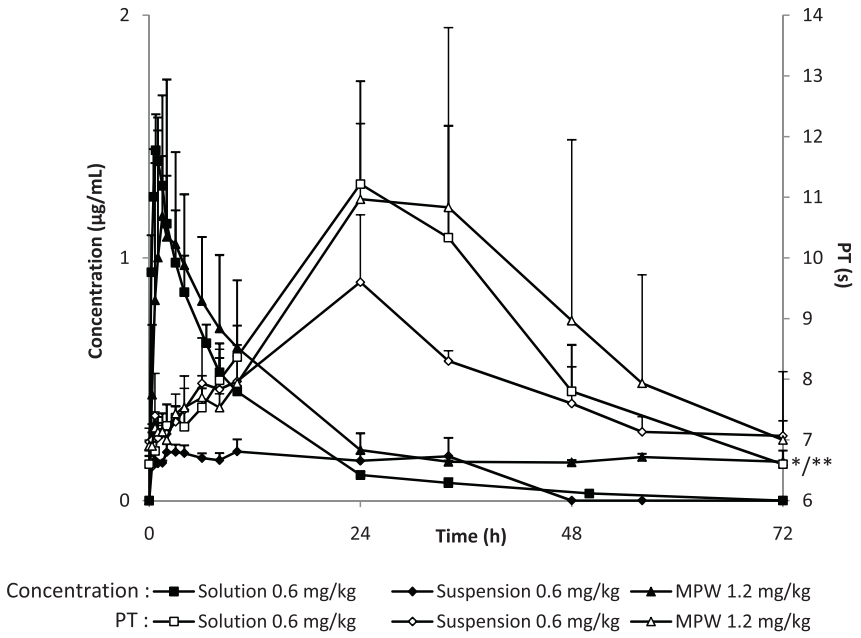

Fig. 3. Plasma concentrations and PT after subcutaneous administration of warfarin in solution, suspension and microparticles to rabbits. Data are shown as mean ± S.D. ($n = 3$). *: statistically different at 20, 40 min and 24, 34, 48, 56, 72 h compared with solution form ($p < 0.05$). **: statistically different at 20 min and 1, 2, 3, 4, 6, 8, 10, 48, 56, 72 h compared with the suspension form ($p < 0.05$).

experiments was expected because warfarin base is a poorly water soluble drug (Class II of biopharmaceutical classification system (Lindenberg et al., 2004)). In contrast, it is interesting to observe the high bioavailability with warfarin microparticles, especially given the *in vitro* results. Thus, it appears that *in vivo* release is a more complicated process which cannot be modelled only by *in vitro* release profiles under sink conditions. It could be concluded that the lower particles size of warfarin powder may improve the *in vitro* release. However, this effect is not observed *in vivo*. This may be due to either aggregation of particles of warfarin powder after subcutaneous administration or the ability of the microparticles to remain individualized in the subcutaneous environment, increasing the specific surface area and allowing better solubilization of warfarin.

After the administration of warfarin, there is a latent period of at least 6–24 h before most of the vitamin K-dependent coagulation factors are eliminated from the blood, while prothrombin (factor II) presents a longer half-life (60–72 h) (Ansell et al., 2008). In clinical practice, the anticoagulant effect of warfarin is monitored by determining the international normalized ratio (INR); defined as the ratio of patient's prothrombin time (PT) to that of the standard PT raised to the power of the ISI (international sensitivity index) of the thromboplastin used. This monitoring of biological activity is important for the adjustment of the warfarin dose. For this reason, the warfarin response was also followed by PT monitoring in our study, in parallel with the plasma concentrations. Previous studies on warfarin release systems (Tarr et al., 1997) (Zacchigna et al., 2004) did not include this double monitoring. As expected, the highest prothrombin times (PT_{max}) were reached at 24 h and were 11.22 ± 1.70, 9.60 ± 1.10 and 11.00 ± 1.30 s for the solution, the suspension and the microparticles, respectively (Table 3). As shown by the biological activity profile (Fig. 3), no large change in the initial PT values was observed between 0 and 8 h. The warfarin concentrations and PT values obtained after microparticles administration were extremely variable, which could be explained by the small number of animals. In addition, the studies led by Sun et al. (2006), Lombardi et al. (2003), or Kulkarni et al. (2008) concluded that INR values were poorly correlated with plasma concentrations of warfarin, which raises the question of which parameter: plasma concentration or biological activity, is the more relevant method for evaluating the *in vivo* performance of new delivery systems. In the clinic, INR monitoring alone is used to adjust the therapeutic dose of warfarin. However, during the development of a new delivery system, the simultaneous determination of plasma concentrations and biological activity as INR or PT could be helpful. Furthermore, as suggested in the study of Sun et al. (2006), the warfarin response can be monitored by the INR/7-hydroxycoumarin concentration ratio as well as by INR or plasma concentrations. Finally, the concentration profiles obtained after administration of microparticles and warfarin solution were statistically different from 24 to 72 h. However, although the plasma concentration of warfarin was higher between 24 and 72 h after microparticles (0.16 µg/mL) than after the solution, no difference in biological activity was observed.

Table 3
Pharmacokinetic parameters of warfarin formulations after subcutaneous injection to fasted rabbits at 0.6 mg/kg for solution and suspension and at 1.2 mg/kg for microparticles. Data are expressed as mean ± SD ($n = 3$).

Calculation based on	Parameters	Warfarin solution	Warfarin suspension	Warfarin microparticles
Plasma concentration	T_{max} (h)	0.75	3.00	1.50
	C_{max} (µg/mL)	1.44 ± 0.20	0.20 ± 0.03	1.17 ± 0.50
	$AUC_{0\ to\ 72h}$ (h µg/mL)	12.16 ± 0.71	7.34 ± 1.67	22.50 ± 5.63
	F_{72h} (%)	100.0	54.2 ± 18.8	92.1 ± 18.6
Biological activity	T_{max} (h)	24	24	24
	PT_{max} (s)	11.22 ± 1.70	9.60 ± 1.10	11.00 ± 1.30
	$AUC_{0\ to\ 72h}$ (h s)	115.8 ± 43.7	71.6 ± 28.4	142.3 ± 126.1
	F_{72h} (%)	100.0	61.8 ± 24.5	61.4 ± 54.5

Although microparticles produced higher plasma concentrations resulting in higher values of AUC and bioavailability than the suspension, no statistically differences were observed in biological activity. These preliminary results suggest that on one hand, an early important peak of concentration is not necessary for the biological activity and on the other hand, a concentration higher than 0.20 μg/mL seems necessary for a sustained biological activity.

Finally, it is quite difficult to compare our results with the scientific literature because there are only a limited number of studies reporting the development of a sustained release system of warfarin. However, it is interesting to notice that after oral administration of warfarin sodium at a dose of 0.54 mg/kg to rabbits, Zacchigna et al. (2004) observed that warfarin remained in plasma for 48 h with a maximal plasma concentration of about 1.70 μg/mL at 3.4 h, corresponding to an approximate mean AUC of 20.0 h μg/ml. It is clear that this estimation of AUC is limited by methodological bias but it allows a comparison of our delivery systems of warfarin with the dosage form of warfarin used in clinical practice. Thus, the relative bioavailabilities for subcutaneous warfarin suspension or microparticles, calculated with respect to oral warfarin sodium powder, were close to 33.0 and 50.4%, respectively, highlighting the influence of the route of administration. In comparison with oral warfarin sodium (the classical treatment), a subcutaneous warfarin suspension or microparticles would avoid the drawbacks of oral route of administration and allow the use of a smaller dose due to a more favourable pharmacokinetic profile characterized by a less intense plasma peak concentration and a more sustained plasma concentration.

4. Conclusion

In this preliminary work, we showed the possibility of preparing a warfarin suspension and warfarin-loaded PCL or PCL/PVP microparticles. The addition of PVP to the formulation allowed the *in vitro* release of warfarin to be modulated. Microparticles containing warfarin would have to be optimized in order to increase the amount of released warfarin after 24 h and to allow an extended biological activity, as estimated through the measurement of PT, the important parameter in clinical practice.

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