

Improvement of warfarin biopharmaceutics by conjugation with poly(ethylene glycol)

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Abstract

One of the most used and useful polymers, poly(ethylene glycol) (PEG) was used as a carrier for warfarin. The drug–polymer conjugate was freely water soluble at room temperature. The hydrolytic stability of the PEG–warfarin was investigated at physiological pH and confirmed the stability of the conjugate. In vivo release studies demonstrated a good release of parent drug, without the initial high plasma level of warfarin.

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1. Introduction

The conjugation of simple drugs to synthetic polymers is of great interest for pharmacological applications. Prodrug design comprises an area of drug research that is concerned with the optimization of drug delivery. A prodrug is a biologically inactive derivative of a parent drug molecule that usually requires an enzymatic transformation within the body in order to release the active drug and has improved delivery properties over the parent molecule. Too rapid a breakdown of the prodrug can lead to spiking of the parent drug and possible toxicity, while too slow a release rate can compromise the drug's efficacy (Greenwald et al., 2003).

Poly(ethylene glycol) or PEG, is one of the most used and useful polymer, since it endows many advantageous features both from a physico-chemical as well as from a biological point of view. Indeed, PEG is amphiphilic and dissolves in organic solvent as well as in water; it is non toxic and eliminated by a combination of renal and hepatic pathways thus making it ideal to employ in pharmaceutical applications.

The FDA has approved this polymer for human intravenous, oral and dermal applications. In the case of PEG conjugates it is clear that the solubility of the prodrug will almost always exceed that of the original drug, usually overcoming any existing aqueous insolubility and thus increasing the possibility of more effective drug delivery. The rate of drug release will be dependent on the nature of the polymer–drug linkage (Greenwald et al., 2003).

Warfarin (Fig. 1) is the most widely used coumarin anticoagulant and is often considered as a drug of choice. If warfarin is employed for a long term anticoagulant therapy, it takes about 3 days for the prothrombin time to return to normal, after drug discontinuation. Warfarin is equally effective either orally or intravenously, but is usually given by mouth. Theoretically sudden discontinuation of warfarin may result in rebound hypercoagulability with risk of thrombosis and therefore some clinicians tail off long-term treatment over several weeks (AA.VV., 1999).

Warfarin is practically insoluble in water and it is administered as warfarin sodium or warfarin sodium clathrate (Babhair et al., 1985). Warfarin is a drug with an elimination half-life of 37 h, which is sufficiently sustained in the body with conventional doses, so controlled release is generally not necessary. Controlled release of drugs that have

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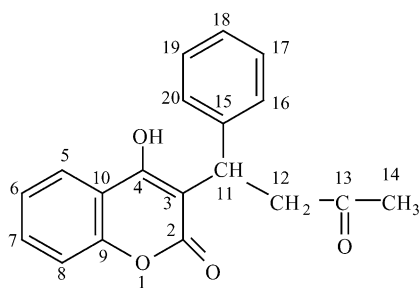


Fig. 1. Warfarin.

long biological half-life is difficult to rationalize. The most plausible reason is that by reducing the absorption rate it is possible to prevent the occurrence of high peak drug levels shortly after dosing and, therefore, the risk of adverse effects. The current active interest in controlled drug release, together with improved technology, has given rise to a rush of new formulations (Welling and Dobrinska, 1987).

As a part of our search for prodrug design (Zacchigna et al., 2002, 2003), we report herein the design, synthesis and evaluation of a macromolecular prodrug of warfarin, a well known anticoagulant drug. The attachment of warfarin to PEG, as a macromolecular carrier, was performed by a covalent bond through succinic acid as spacer group. Furthermore, *in vitro* and *in vivo* studies were carried out in order to evaluate the behaviour of the obtained prodrug. While several studies have been reported on pharmacokinetics (Smith et al., 2000a,b; Stangier et al., 2000) and formulation (Ammar et al., 1997) of warfarin this is the first report on pegylation of this anticoagulant drug.

2. Materials and methods

2.1. Chemicals

Warfarin (3-(α-acetonylbenzyl)-4-hydroxycoumarin), warfarin sodium (3-(α-acetonylbenzyl)-4-hydroxycoumarin sodium salt), chloro warfarin ((±)-3-(α-acetonyl-*p*-chlorobenzyl)-4-hydroxycoumarin), triethylamine (TEA) were obtained from Sigma Chemicals Co. (St. Louis, MO, USA).

4-Dimethylaminopyridine (DMAP), poly(ethylene glycol) of MW = 2000 ($n = 43$), succinic anhydride, dicyclohexylcarbodiimide (DCC) were purchased from Fluka.

All the other chemicals and solvents used in this work were of the highest quality commercially available. Organic solvents were dried over molecular sieves (3 Å).

2.2. Laboratory animals

Male rabbits weighing 5 kg free from any signs of abnormality were used in this study (Charles, River, Calco, Italy).

2.3. Analytical methods

Infrared spectra were recorded using a Perkin-Elmer 1720 IR Fourier transform spectrophotometer. High-performance liquid chromatographic (HPLC) analyses were carried out with a Perkin-Elmer series 4-high-performance liquid chromatography, equipped with a Rheodyne Model 7125 injector with a 20 μl loop and connected to a Perkin-Elmer Model LC 75 variable-wavelength UV detector. A LiChrosorb RP18 (Perkin-Elmer) 250 mm × 4.6 mm i.d. column packed with 5 μm particle size was used.

The purity of the synthesized compounds was checked by TLC on precoated silica gel aluminium sheets (Merck 60 F₂₅₄). Spots were visualized under 254 nm illumination.

Potentiometric titrations were carried out using a TTT80 pH stat equipped with an ABU80 autoburette, a titrgraph module REA 160 and a REA 070 pH stat unit, radiometer. Elemental analyses were carried out on a Carlo Erba model 1016 analyser. ¹H NMR and ¹³C NMR spectra (CDCl₃) were recorded on a Varian Gemini-200 MHz spectrometer.

¹³C NMR: 100.4 MHz. Chemical shifts are given in ppm from TMS as internal standard. The following abbreviations are used: s, singlet; br s, broad singlet; d, doublet; t, triplet; m, multiplet; respectively.

2.4. Preparation of PEG–COOH

PEG₂₀₀₀ (10.5 g, 0.01 mol of OH groups) was dissolved in toluene (100 ml) and dried by distilling off most of the toluene. Succinic anhydride (4.2 g, 0.04 mol) was added and the mixture was stirred for 5 h in an oil bath at 150 °C. The mixture was cooled, taken up in CH₂Cl₂ and the polymer precipitated by ether. The product was recrystallized twice from CH₂Cl₂/ether. Yield 90%.

TLC: BuOH/AcOH/H₂O, 4:1:1 v/v.

Analysis: calcd for C₇₄H₁₈₂: C, 40.36; H, 8.27, MW 2200. Found: C, 40.52; H, 8.60.

IR (nujol): $\nu = 1735 \text{ cm}^{-1}$ (C=O), 1100 (C–O).

¹H NMR (CDCl₃): $\delta = 2.60 \text{ ppm}$ (br s; 8H, CH₂COO–), 3.60 (br s; CH₂–O–CH₂), 4.20 (t; 4H, CH₂OCO).

¹³C NMR (CDCl₃): $\delta = 28.2\text{--}30.0 \text{ ppm}$ (CH₂COO), 64.1 (CH₂OCO), 69.0–70.6 (CH₂–O–CH₂), 172.0 (COO), 174.2 (COO).

2.5. Preparation of PEG–warfarin

Warfarin (6.8 mmol; 2.1 g), DCC (9 mmol; 1.86 g) were added in small portions to a solution of PEG₂₀₀₀–(COOH)₂ (4.5 mmol; 5 g) and DMAP (2 mmol; 0.44 g) in CH₂Cl₂ (50 ml); the mixture was stirred for 5 h at room temperature. The precipitate of dicyclohexyl urea (DCU) was filtered off and the filtrate evaporated to dryness. The residue was extracted with CH₂Cl₂, filtered from DCU residue and the product precipitated by ether. The product was recrystallized twice from ethanol. Yield 85%.

Analysis. Calcd for C_{132} ; H_{210} : C, 56.97; H, 7.55, MW 2780; found: C, 56.82; H, 7.80.

^1H NMR ($CDCl_3$): δ = 2.06 ppm (s; 6H, CH_3CO), 2.61 (br s; 8H, CH_2COO), 3.46–3.60 (br s; CH_2COCH_3 , CH_2-O-CH_2), 4.0 (t; 2H, $CHPh$), 4.20 (t; 4H, CH_2OCO), 7.2–7.9 (m; 18H, arom-H).

^{13}C NMR ($CDCl_3$): δ = 216.5 ppm (C-13), 176.9 (C-2), 171.9 (COO), 170.1 (COO), 160.7 (C-4), 152.9 (C-10), 142.4 (C-3), 131.9 (C-15), 128.8 (C-16, C-20), 127.0 (C-17, C-19, C-7), 126.78 (C-18), 123.9 (C-6), 122.9 (C-5), 116.5 (C-8), 104.9 (C-9), 69.0–70.6 (CH_2-O-CH_2), 64.0 (CH_2-OCO), 41.7 (C-12), 35.15 (C-11), 28.9–29.8 (CH_2COO), 24.4 (C-14).

2.6. *In vitro* drug release studies

An HCl, NaCl and glycine buffer (pH 1.2, 0.2 M), a phosphate/citrate buffer (pH 5.5, 0.2 M), and a phosphate buffer (pH 7.4, 0.1 M) were used. To investigate the hydrolytic stability of PEG–warfarin. 20 mg/ml was dissolved in three buffers (pH 1.2, pH 5.5 or pH 7.4), containing an appropriate quantity of internal standard at $37 \pm 0.1^\circ C$. Samples were taken at suitable intervals and the release of warfarin was measured with HPLC.

2.7. *In vivo* drug release studies

Warfarin sodium (0.54 mg/kg body weight) and PEG–warfarin (2.3 mg/kg body weight) were solubilized in physiological solution and administered orally, intravenously or intramuscularly at three rabbits.

Blood (1.5 ml) was withdrawn immediately prior to drug administration. Further 1.5 ml blood samples were obtained at suitable intervals of time after drug administration. The plasma was separated by centrifugation. Warfarin concentrations in plasma were measured with HPLC. The sample was prepared following a method already known in literature (Bjornsson et al., 1977). Briefly: to 500 μ l of plasma in a capped test tube were added 500 μ l of sulphuric acid 0.5 N containing 100 μ l of *p*-chloro warfarin (1 mg/ml) as internal standard and 5 ml of ether. After vortexing for 60 s, the samples were centrifuged for 5 min at 10,000 rpm. Four milliliter of the organic layer were transferred into a glass tube, and evaporated to dryness under a nitrogen stream. The residue was reconstituted with 50 μ l of the methanol, and 20 μ l of the solution were injected in HPLC.

2.8. HPLC analysis

Reverse phase HPLC C18 column was used. A mixture of methanol and water (85:15 v/v %) pH 5 was employed as mobile phase, and the detection wavelength was 308 nm. *p*-Chloro warfarin was used as internal standard.

3. Results

3.1. Preparation of PEG–warfarin

A PEG–warfarin conjugate was synthesized in order to study the role of PEG in drug delivery. An ester bond links PEG to warfarin. Esters with PEG as an electron-withdrawing substituent (alkoxy) in the α -position proved to be especially effective linking groups in the design of prodrugs since they aid in the enzymatic hydrolysis of the ester carbonyl bond, and thus are able to release alcohols in a continuous and effective manner (Greenwald et al., 2003).

For the synthesis of warfarin prodrug, PEG₂₀₀₀ was used. The hydroxylic groups of polymer were activated to carboxylic groups by succinic anhydride (90%) (Zalipsky et al., 1983). TLC showed only one spot and no succinic anhydride. The product titrated with 0.01 N NaOH yielded 100% of free carboxylic groups.

The attachment of warfarin to the carboxylated polymer was performed by means of DCC and DMAP with a good yield (85%) (Fig. 2). The absence of free drug was verified by HPLC analysis.

^1H NMR and ^{13}C NMR analysis were useful in providing informations for the structural elucidation of the synthesized drug-polymer conjugate.

The ^1H NMR spectrum of the adduct indicated the presence of methylene protons ascribed to PEG moiety (δ = 3.46–3.60 ppm), while the chemical shifts of the aromatic protons of warfarin were observed at δ = 7.20–7.90 ppm. The signals at δ = 171.9 ppm and 170.1 ppm in the ^{13}C NMR spectrum of the conjugate confirmed the ester bond between the drug and the activated polymer.

The percentage of linked drug, evaluated by alkaline hydrolysis followed by HPLC analysis, was estimated equal to 100% (w/w).

3.2. *In vitro* release studies

In order to gain some preliminary information about the potential use of PEG–warfarin as a drug delivery system the conjugate was subjected to hydrolysis at $37 \pm 0.1^\circ C$ in buffer solutions at physiological pH, simulated gastric juice, pH 1.2, endosomal compartments, pH 5.5, and extracellular fluids, pH 7.4.

Samples were regularly taken out of the incubation mixture and the percentage of released warfarin was quantified with the HPLC. Each experiment was carried out in triplicate and expressed as the mean value $\pm$ S.D. The results are depicted in Fig. 3.

The amount of warfarin released from the polymeric conjugate at pH 7.4 in phosphate buffer solution was 2.5% over 24 h. In buffer solutions pH 5.5, and 1.2 less than 1.5% of warfarin was released over 24 and 6 h, respectively. The conjugate therefore was hydrolytically stable in buffers under physiological conditions.

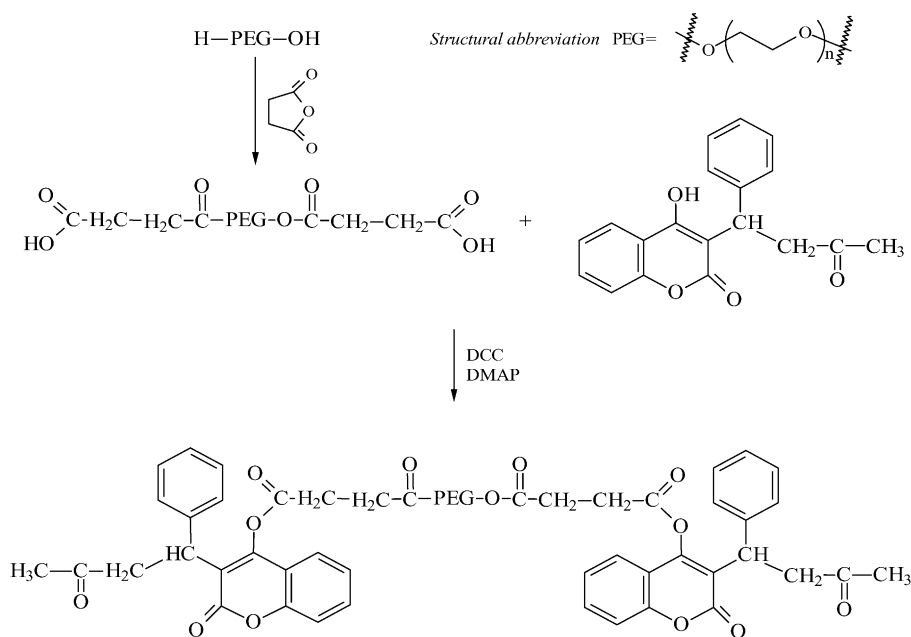


Fig. 2. Synthesis of PEG-warfarin.

3.3. In vivo release studies

In order to evaluate the biological performance of the adduct, a physiological solution of PEG warfarin, containing an amount of conjugate giving a dose of 0.5 mg/kg body weight of warfarin, was administered in three separate ways: orally, through a gastric tube and parenterally. Three male rabbits fasted for 12 h prior to use, were used as subjects. The group of animals was treated in the same way with a solution of warfarin sodium (0.54 mg/kg body weight) as control drug. Each drug solution was prepared immediately prior to use and the administration was carried out at a distance of 21 days in order to guarantee the complete elimination of warfarin from the body. The blood samples were taken from the right ear of the rabbits at regular time intervals. The plasma was separated conventionally (Gasic et al., 1968) and stored in a freezer pending assay.

The warfarin present in the plasma samples was quantified by HPLC and the analysis of drug was validated. The limit of quantification was approximately 0.1 $\mu\text{g}/\text{ml}$. The coefficient of variation calculated for the six identical samples analyzed did not exceed 5%. The precision of the assay method was calculated by determining the relative standard deviations of peak height ratios obtained from six replicate assays within a concentration interval of 0.1–10 $\mu\text{g}/\text{ml}$. The relative standard deviation ranged from 1 to 5% for intraday analysis and from 2 to 6% for interday analysis. The absolute recoveries of warfarin and the internal standard in plasma were determined by comparing the slopes of the processed human plasma standard curves and the standard curves prepared in methanol. The recoveries of warfarin and the internal standard were $82 \pm 4\%$ and $84 \pm 6\%$, respectively.

Warfarin plasma concentration–time curves after oral and parenteral administrations of a physiological solution of

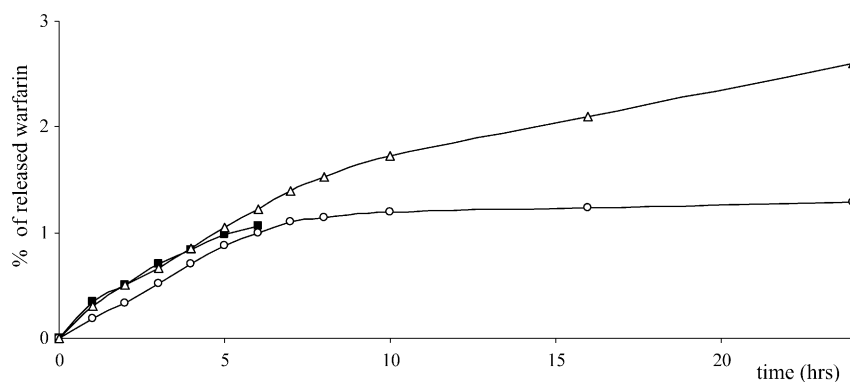


Fig. 3. Release of warfarin from PEG-warfarin in buffer solutions, at 37°C: (■) pH 1.2 0.2 M HCl, NaCl, and glycine, (○) pH 5.5 0.2 M phosphate/citrate, and (△) pH 7.4 0.1 M phosphate.

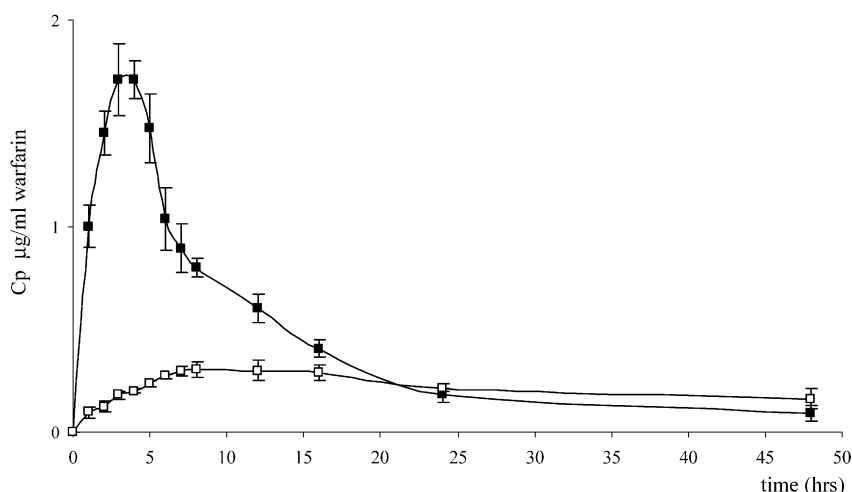


Fig. 4. Plasmatic concentration of warfarin after oral administration of (■) warfarin sodium and (□) PEG-warfarin. Each value is the mean $\pm$ S.D. for three rabbits.

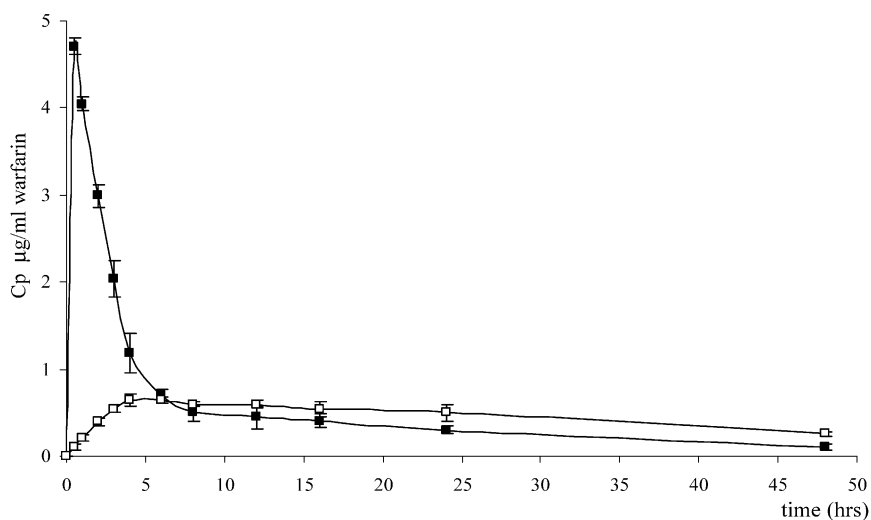


Fig. 5. Plasmatic concentration of warfarin after intramuscular administration of (■) warfarin sodium and (□) PEG-warfarin. Each value is the mean $\pm$ S.D. for three rabbits.

macromolecular prodrug and of warfarin sodium are reported in Figs. 4–6.

Warfarin sodium gives a plasma concentration of drug that immediately increases after administration, but decreases very rapidly with time and remains fairly constant. The drug plasma concentration after administration of the polymeric prodrug is initially lower when compared with the administration of free drug and decreases very slowly with time, remaining practically constant for about 40 h.

4. Discussion

Treatment with oral anticoagulants must be monitored to ensure that the dose is providing the required effect on the Vitamin-K-dependent clotting factors: too small a dose pro-

vides inadequate anticoagulation, too large a dose puts the patients at risk of haemorrhage. This monitoring is commonly carried out by checking the prothrombin time. Measurement should be carried out before treatment and then daily or on alternate days in the early stages of treatment. Once the dose has been established and the patient well stabilized the measurement can be made at greater, but regular, intervals. Dosage must be determined individually. A usual initial dose of warfarin sodium is 10 mg daily by mouth for 2 days, but may be lower for patients at particular risk of haemorrhage. Subsequent maintenance doses usually range from 3 to 9 mg/die. These doses give steady state plasma concentration of 0.72–2.9 μ g/ml.

In this paper, the synthesis of a polymeric warfarin conjugate was described. Warfarin was covalently linked to the macromolecular carrier, poly(ethylene glycol), via ester formation.

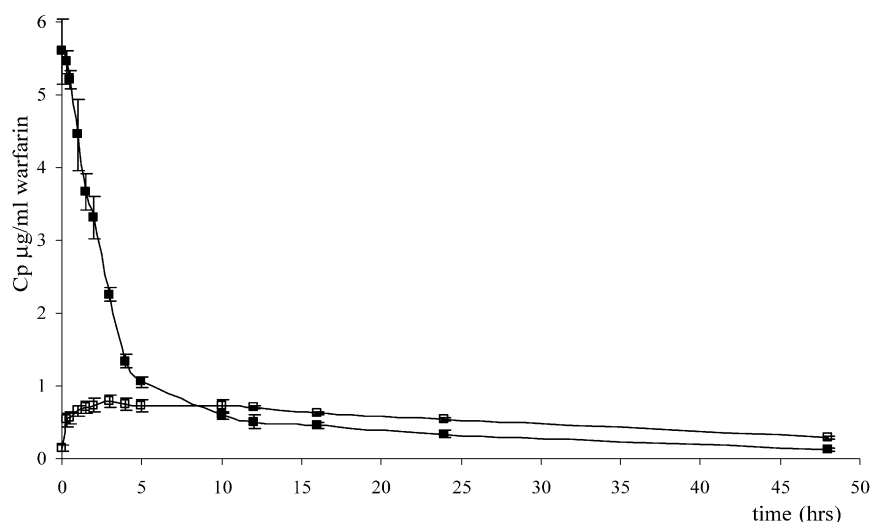


Fig. 6. Plasmatic concentration of warfarin after i.v. administration of (■) warfarin sodium and (□) PEG-warfarin. Each value is the mean $\pm$ S.D. for three rabbits.

The PEG-warfarin prodrug showed some interesting peculiarities which make it attractive in the drug delivery for anticoagulant treatment. It was very soluble in water and stable in physiological buffer, but, in vivo, it was able to release drug in a constant and effective manner. This is the first report of a PEG-warfarin prodrug.

As the ratio of warfarin in the macromolecular conjugate is almost 25%, 12–36 mg/die doses of the prodrug could give the same plasma concentration at the steady state, without the high drug concentration found immediately after the administration of warfarin sodium. As the fluctuation of the concentration at steady state is reduced and the therapeutic concentration is more linear and constant, the risk of adverse effects, like haemorrhage, can be reduced. In such cases the checking of the prothrombin time could be less frequent and consequently the compliance of the patient could be increased.

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