Bioorganic Chemistry 54 (2014) 1–6

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Development and evaluation of PEGylated Enoxaparin: A novel approach for enhanced anti-Xa activity

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ARTICLE INFO

Article history:
Received 19 December 2013
Available online 11 March 2014

Keywords:
Enoxaparin
Low molecular weight heparin
Venous thromboembolism
Polyethylene glycol conjugate
Anti-Xa activity

ABSTRACT

Enoxaparin (ENX) is one of the most widely prescribed low molecular weight heparin in prophylaxis and treatment of venous thromboembolism. In this study, Enoxaparin–PEG conjugate (P-ENX) was synthesized from Enoxaparin and polyethylene glycol (PEG) and evaluated for its potential for extended duration of action. The esterification of the carboxyl groups of the drug moiety with the hydroxyl groups of mPEG-2000 was done by employing carbodiimide coupling chemistry. P-ENX conjugate was purified by dialysis and characterized by Fourier transform infrared spectroscopy (FTIR), Proton-Nuclear magnetic resonance (^{1}H NMR) and matrix-assisted laser desorption/ionization (MALDI) mass analysis techniques. FTIR analysis revealed frequency of the carbonyl group in accord with ester linkage formation between the drug and the PEG moiety. ^{1}H NMR of the conjugate showed significant change in the chemical shift further indicative of ENX and PEG chemical interaction. In MALDI spectra, small peaks at 12,907 and 16,137 *m/z* confirmed the probability of conjugation of ENX and PEG. P-ENX exhibited considerable enhancement in anti-Xa activity (by three-folds) in comparison to free ENX. Further, an increase in AUC (over four-folds) was observed in P-ENX. Thus, PEGylation of ENX is a novel approach for extended and enhanced activity of ENX with a potential for decreased dosing frequency.

1. Introduction

Venous thromboembolism (VTE) is a fatal blood coagulation disorder leading to inappropriate blood clots formation, hence this silent and potential lethal disease results in considerable morbidity and mortality. VTE includes both deep-vein thrombosis (DVT) – clots are formed in the deep veins of the body; and pulmonary embolism (PE) – a clot enters the arteries of lungs [1,2]. Approximately; one-third of VTE patients develop PE while two thirds develop DVT. There is an increase in incidence from 50 to 100 in the young to over 500 per 100,000 in the elderly [2,3]. The most common predisposing factors of VTE are malignancy (31%) and post-operative status (30%) [4]. Ideally, prevention and immediate treatment are of paramount importance in order to reduce the number of deaths, hospitalizations, and the cost of therapy [5]. The initial aim of DVT treatment is to prevent thrombus formation and PE, and the long-term goal is to decrease the incidence of recurrent VTE. A variety of drugs such as un-fractionated heparin (UFH), low molecular weight heparins (LMWHs) and warfarin, affecting coagulation cascade are being used to manage VTE. Unfortunately, none of the existing anticoagulant therapies are optimal with regard to ease of administration, safety, and efficacy.

Over the past few years, LMWHs have received widespread acceptance as the drug of choice for the short-term treatment of VTE [6] as they offer several advantages over un-fractionated heparin, including a more predictable dose–response relationship, lower risk of heparin-induced thrombocytopenia, and reduced overall cost [7]. However, there are some important limitations that preclude the use of LMWH as a maintenance therapy for the disorder. Firstly, similar to un-fractionated heparin, LMWHs still require painful subcutaneous administration. Secondly, LMWHs have a relatively short duration of action, necessitating once or twice daily injection. ENX is one of the most widely used LMWH in the prophylaxis and treatment of VTE [8].

PEGylation is one of the most extensively used techniques for enhancing the efficacy of pharmaceuticals. A variety of therapeutic agents have been reinvented by PEGylation as markedly intensified treatments with an extended circulating half-life, reduced

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http://dx.doi.org/10.1016/j.bioorg.2014.03.002
0045-2068/

immunogenicity, increased stability, and improved *in vivo* bioactivity [9–16]. A long-acting LMWH via a noninvasive administration route as a replacement therapy in VTE would significantly improve treatment compliance for patients.

In this study, with an aim to extend ENX's duration of action, thereby reducing its frequency of administration, conjugation with medium molecular weight polyethylene glycol followed by confirmation of anti-Xa activity as compared to pure ENX has been reported. An increase in the duration of its anti-Xa activity, thus reflecting on enhancement of its half-life, has been observed [3].

2. Results and discussion

In this study, P-ENX was synthesized by the esterification of the carboxyl groups of ENX moiety with the hydroxyl group of mPEG at room temperature. A well-known water soluble carbodiimide, EDAC was used as the coupling agent, with DMAP as a base catalyst. EDAC is a versatile coupling agent to form amide, ester or thioester bonds and thus, to cross-link proteins, nucleic acids and/or to bind molecules to surfaces in aqueous or organic media. EDAC was selected instead of dicyclohexylcarbodiimide (DCC) because of the aqueous reaction conditions. EDAC exhibits enhanced solubility in water (>200 g/L) and in organic solvents along with ease of use. EDAC hydrochloride was preferred because of its long-term stability, non-hygroscopicity and its resistance to hydrolysis [17]. DMAP is also a well-known catalyst for the esterification of alcohols by acid anhydrides and other synthetically useful transformations involving an acyl transfer. It is observed that addition of 3–10 mol% DMAP accelerates the DCC or EDAC-activated esterification of carboxylic acids with alcohols or thiols to such an extent that formation of side products is suppressed and even sterically demanding esters can be obtained at room temperature [18].

Firstly, the sodium salt of ENX was converted into its free acid form. The by-product, sodium chloride was removed by dialysis. Freeze drying of the solution gave the free acid form of ENX. Secondly, acidic ENX was conjugated with mPEG 2000 by formation of an ester linkage using EDAC and catalytic amount of DMAP. The crude was isolated containing the conjugate, un-reacted drug and mPEG2000 along with by-products.

2.1. Purification

Dialysis was employed for purification of the above obtained crude. The dialysis membrane (MWCO 2000) was selected because the molecular weight (MW) of conjugate was above 2000 and other contaminants such as EDAC. HCl (MW-191.74), DMAP (MW-122.17) and by-product (MW-173) had lower MW. After dialysis, the product was obtained, free from of low molecular weight impurities. Un-reacted mPEG obtained was removed from the reaction mixture by considering its solubility difference-mPEG is soluble in DCM while the conjugate is sparingly soluble. The freeze-dried product was suspended in DCM. The conjugate remained suspended and was isolated from ENX. This process was repeated three times for complete removal of mPEG from P-ENX. The product was concentrated on rotary evaporator, dissolved in water and freeze dried to obtain P-ENX. As ENX is very polydisperse in nature having a range of molecular weights from less than 2000 to more than 8000, P-ENX will also be polydispersed. The product subsequently was characterized by FTIR, ^{1}H NMR and MALDI spectroscopic methods.

2.2. Characterization

The characteristic peaks present in FTIR spectra (see Fig. 1) of pure ENX at 3445 cm^{-1} corresponded to O—H stretching of hydroxyl groups, 1622 cm^{-1} for C=O stretching of carboxyl group, 1240 cm^{-1} for S=O stretching of sulfate group and 995.21 cm^{-1} for C—O—C stretching of ethereal linkage. FTIR spectra of mPEG 2000 contained apeak at 3422 cm^{-1} for O—H stretching, 2885 cm^{-1} for C—H stretching of repeating ethylene groups. After esterification, i.e., in P-ENX, the peak at 1622 cm^{-1} was expected to shift to higher frequency as ester's exhibit enhanced C=O stretching than acids due to increase in double bond character of the carbonyl group. The C=O stretching peak had shifted to 1633 cm^{-1} indicating the formation of ester bond in the conjugate. In addition, larger and sharper aliphatic stretching band of C—H was appeared at 2889 cm^{-1}, due to the possibly increased number of C—H bonds by conjugation with mPEG.

^{1}H NMR spectrum (see Fig. 2) of ENX exhibited characteristic chemical shifts with a signal at 5.96 ppm corresponding to H-4 of unsaturated nonsulfated uronic acid residue located at the non-reducing end and a peak at 3.26 ppm for 1, 6-anhydro derivative present in 15–25% molecules at the reducing end. In addition, peaks at 5.55 and 4.6 ppm corresponded to H-2 and H-1 protons of uronic acid residue, and peaks at 2.02 and 2.21 ppm corresponded to N-acetyl and acetate group, respectively. Other signals representing anomeric hydrolysis were at 5.43 and 5.49 ppm due to the formation of 1–4 linkage between uronic acid and glucosamine residues, and several other peaks were also present in ^{1}H NMR spectrum and individual peaks were difficult to be assigned due to its complex nature. NMR spectrum of mPEG shows the signal at 3.6 and at 3.4 ppm corresponding to methylene protons and methoxy protons, respectively. No peak was observed for hydroxyl proton in D_2O, but it was present at 4.66 ppm when the spectrum was recorded in DMSO. P-ENX proton spectrum contained all the peaks corresponding to ENX and mPEG, and no firm conclusion can be drawn. The sole difference expected was the disappearance of peak of the hydroxyl proton of mPEG and acidic protons of ENX, due to conjugation. However, the hydroxyl protons were also absent, in the spectra of ENX and mPEG. Thus, NMR spectra could not be relied upon as a direct evidence to confirm the conjugate's structure.

MALDI spectra of mPEG showed average molecular weight of polymer to be 2000. Number average molecular weight (Mn), weight average molecular weight (Mw), and the polydispersity index (PDI) was calculated from the following formulas:

$$\mathrm{Mn} = \frac{\sum \mathrm{NiMi}}{\sum \mathrm{Ni}};\ \mathrm{Mw} = \frac{\sum \mathrm{NiMi}^2}{\sum \mathrm{NiMi}}\mathrm{PDI}$$

The Mn and Mw calculated for mPEG 2000 were 1984.925 and 2021.851 respectively, and PDI was 1.0186. The MALDI spectra, as shown in Figs. 3 and 4 exhibited a difference of 44 units between each peak representing the monomer unit of CH_2CH_2O. The numbers of monomer units present in mPEG were 30–60 with an average value of 45. MALDI analysis of ENX displayed no characteristic peaks while mass analysis of P-ENX gave spectrum representing mPEG. ENX, a large molecule with very high polarity, was difficult to ionize through normal MALDI procedure, and the energy used for ionization resulted in complete fragmentation of the molecule. Therefore, no peaks were obtained in ENX, and the presence of mPEG peaks in the conjugate is an indication of breakage of the ester bond. This provides indirect evidence in favor of the expected structure of P-ENX. A new mass analysis for ENX either MALDI or any other method could potentially be developed in order to complete the characterization of the conjugate.

2.3. Anti-Xa assay method

TECHNOCHROM® anti-Xa assay, a system of reagents for the colorimetric determination of direct and indirect Xa inhibitors in

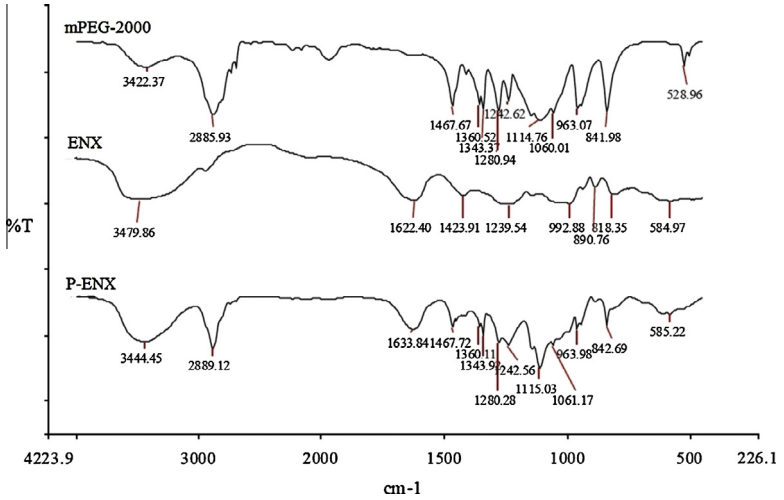

Fig. 1. FTIR spectra of Enoxaparin, mPEG 2000 and Enoxaparin–PEG conjugate.

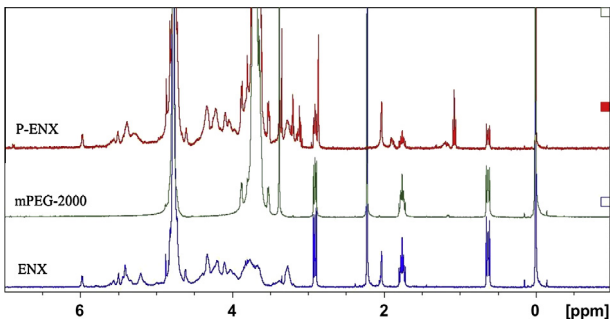

Fig. 2. NMR spectra of Enoxaparin, mPEG 2000 and Enoxaparin–PEG conjugate.

citrated plasma, is the method of choice for measuring heparin and their analogues in the plasma samples. The LMWH anti-Xa assay is a two-step chromogenic method. In stage 1, there is an inhibition of a constant amount of factor Xa by ENX in presence of exogenous antithrombin (AT). In stage 2, there is hydrolysis of a factor Xa-specific chromogenic substrate by the factor Xa in excess leading to release of *p*NA (*p*-nitroaniline) from the substrate. The amount of *p*NA released is proportional to residual factor Xa activity. Thus, there is an inverse relationship between the concentration of heparin and color development, which is measured at 405 nm. The compositions of reagents are provided in Table 1.

- [Xa-Inhibitor] + Xa (excess)———[Xa-Inhibitor – Xa] + Xa (residual).
- Xa (residual) + Substrate———*p*NA.

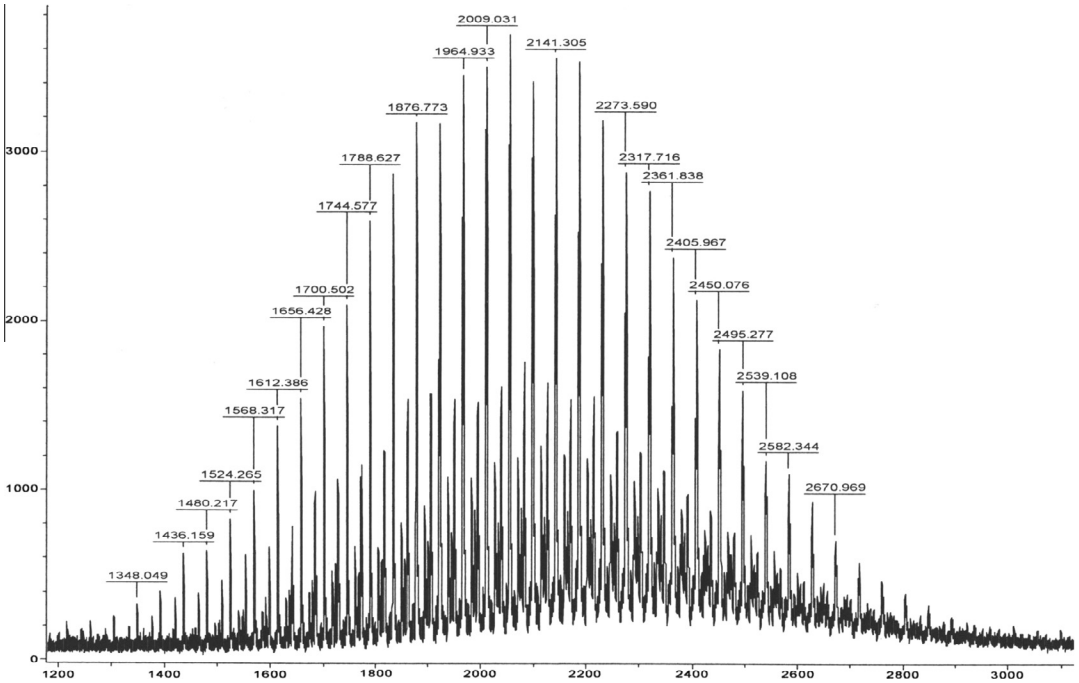

Fig. 3. MALDI spectrum of mPEG2000.

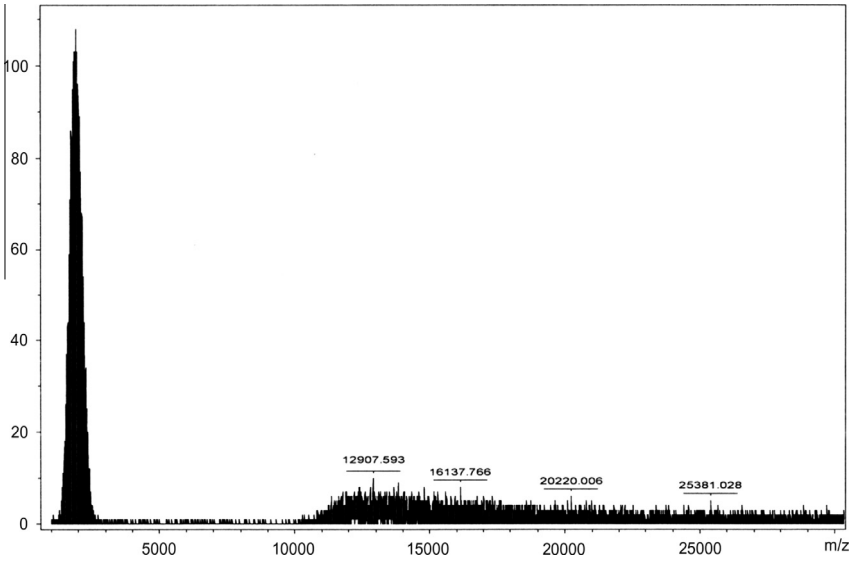

Fig. 4. MALDI spectra of Enoxaparin–PEG conjugate.

Table 1
Composition of reagents employed in determination of anti-Xa activity.

Volume (mL)	Reagents	Description
1 × 20 ml	Reagent 1 (R1)	TRIS-EDTA buffer; pH 8.4; contains sodium azide (<1 g/L) as a preservative
1 × 4 ml	Reagent 2 (R2)	Bovine Factor Xa, lyophilized; 1 vial; about 24 nkat
1 × 4 ml	Reagent 3 (R3)	Chromogenic substrate, lyophilized; 1 vial; 4.8 mg

Calibration curve was prepared for the concentration range of 1 μg/ml (0.1 IU/ml) to 16 μg/ml (1.6 IU/ml) with a correlation coefficient of 0.9653. Calibration data is provided in Table 2, and the calibration graph is presented in Fig. 5.

2.4. Plasma profile and pharmacokinetic study

ENX (4 mg/kg) and P-ENX were administered subcutaneously, and blood samples were collected at time intervals of 0, 0.5, 1.0, 1.5, 2, 4, 6, 12 and 24 h. The blood samples were centrifuged at 2500 g for 15 min, and plasma was separated and stored at −20 °C until analysis. The anti-Xa activity was determined in the plasma by Technochrom anti-Xa kit, and data was expressed in μg/ml. Pharsight WinNonlin software was employed in calculating the pharmacokinetic parameters, and the results are summarized in Table 3. Plasma concentration vs. time profile of P-ENX and pure ENX is presented in Fig. 6. P-ENX exhibited a three-fold enhancement (7.00 vs. 2.61 h) in half-life in comparison to ENX alone. Its peak plasma concentration, C_{max}, was 13.71 μg/ml and T_{max} was 2 h. In pure ENX; the C_{max} was 14.04 μg/ml, and the T_{max} was 1 h. The area under the curve ($AUC_{0-\infty}$) of P-ENX was significantly higher than ENX (147.65 vs. 35.63 μg ml^{-1} h^{-1}). The conjugate was detectable in plasma after 24 h. In contrast, pure ENX was undetectable after six hours. Thus, a significant and a considerable increase in duration of action can be expected from P-ENX. Furthermore, an increase in AUC also indicated the increase in

Table 2
Calibration Parameters.

Parameter	Specifications
Linearity range	1–16 μg/ml
R^2	0.9653
Slope	−0.0176
Intercept	0.5519
LOQ	0.5 μg/ml

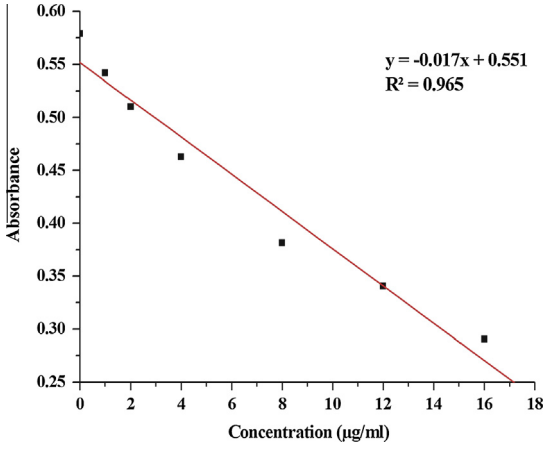

Fig. 5. Calibration curve for anti-Xa assay.

Table 3
Pharmacokinetic parameters of Enoxaparin and the PEGylated Enoxaparin.

Pharmacokinetic parameters	Enoxaparin	Enoxaparin–PEG
$t_{1/2}$	2.6051 h	7.0026 h
C_{max}	14.0349 μg/ml	13.7151 μg/ml
T_{max}	1 h	2 h
$AUC_{0-\infty}$	35.6352 μg ml^{-1} h^{-1}	147.6512 μg ml^{-1} h^{-1}
Vz	0.09 L	0.0615 L
Cl	26.0051 ml/h	6.0852 ml/h

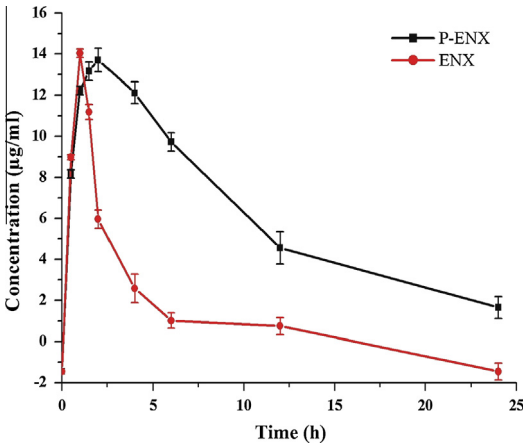

Fig. 6. Plasma concentration–time profiles of Enoxaparin and Enoxaparin–PEG conjugate.

overall bioavailability of the drug in the conjugate. The rate of clearance and volume of distribution were decreased in P-ENX justifying its longer duration of action in comparison to ENX. Fig. 7 presents the pharmacokinetic parameters of ENX and its PEGylated conjugate. Paired *t*-test showed a significant difference between plasma concentration vs. time profiles of ENX and P-ENX with a *p*-value of 0.0172. In addition, considerable difference was observed in the plasma concentrations at each time point.

3. Methodology

3.1. Materials used

ENX sodium was a generous gift from Strides Arcolab Limited (Bangalore, India). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) was purchased from Himedia Laboratories Pvt. Ltd. (Mumbai, India). Dialysis membrane (MWCO 2000), 4-dimethylamino pyridine (DMAP) and poly (ethylene glycol) methyl ether (mPEG 2000) were purchased from Sigma Aldrich (St. Louis, USA). Dimethyl formamide (DMF) was purchased from Merck (Mumbai, India). All other reagents used were of analytical grade, commercially available, and were used without further purification.

3.2. Conversion of ENX Sodium to ENX Free Acid Form

To an aqueous solution of ENX sodium, 1 M HCl was added drop wise until pH 3 was attained. After 5–10 min, sodium chloride formed was removed by dialysis (MWCO 2000) for 24 h in

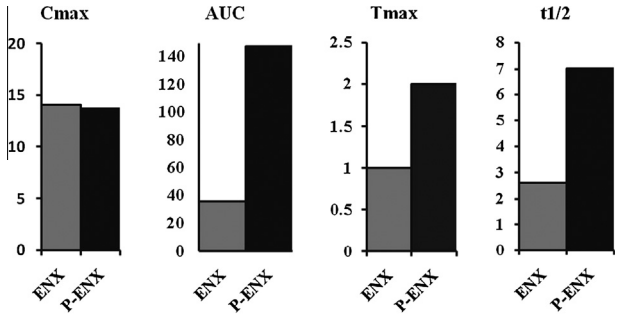

Fig. 7. Comparison of pharmacokinetic parameters of Enoxaparin and Enoxaparin–PEG conjugate.

de-mineralized water. The above solution was freeze-dried to obtain free acid of ENX.

3.3. PEGylation of ENX

ENX was PEGylated with mPEG2000 by using a reported method in the literature [19]. To a solution of mPEG 2000 (2000 mg, one mM) and EDAC (76.68 mg, 0.4 mM) in DMF, DMAP (12.2 mg, 0.1 mM) was added followed by ENX solution (0.2 mM). The obtained mixture was refluxed for 12 h, followed by concentration on a rotary evaporator (BUCHI, Switzerland) to remove DMF. The synthesis of P-ENX conjugate is depicted in Fig. 8.

3.4. Purification of P-ENX

P-ENX was purified by dialysis. An aqueous solution of the crude product, from the previous step, was subjected to dialysis in water for over 48 h, and then freeze dried. The freeze-dried product was again dissolved in dichloromethane to remove mPEG and ENX from P-ENX conjugate.

3.5. Characterization

Infrared spectroscopy was performed by fabricating potassium bromide pellets of ENX and P-ENX conjugate in FTIR (Perkin Elmer Synthesis Monitoring System) equipped with Spectrum-1 software. ^{1}H NMR spectroscopy was recorded in D_2O employing ZH079807 Bruker 400 UltraShield™ spectrophotometer (with Bruker B-ACS120 Autosampler and Topspin 2.7 software). The mass distribution of drug, mPEG and the conjugate were obtained from MALDI mass spectrometry. MALDI spectra were recorded on Ultraflex TOF/TOF instrument (Bruker Daltonics) equipped with Flax control programme and analysis software. Reflectron mode analysis was conducted at 25 kV in negative ion mode with di-hydroxy benzoic acid (DHB) as the matrix.

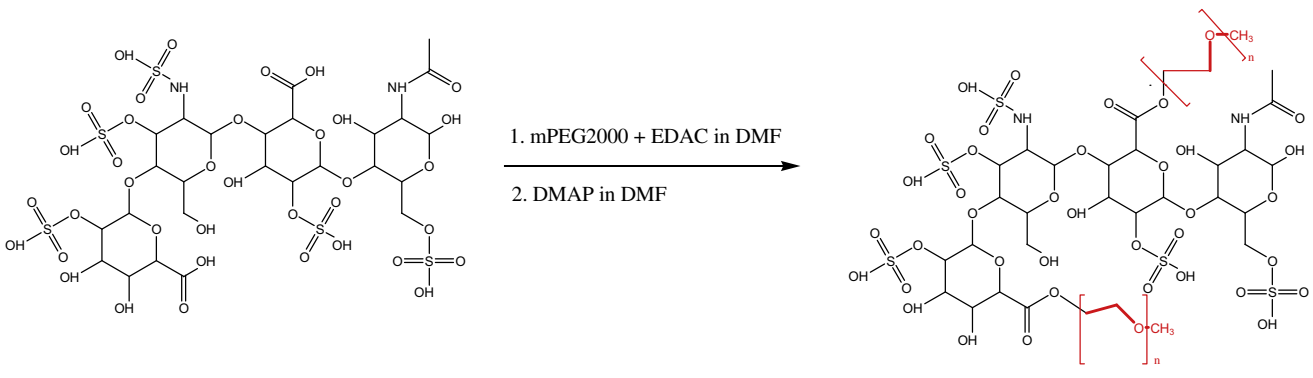

Fig. 8. Scheme of synthesis of Enoxaparin–PEG conjugate.

3.6. Pharmacokinetic study

Female Sprague Dawley (SD) rats (body weights of 160–180 g and 5–6 weeks old) were supplied by the central animal facility (CAF), National Institute of Pharmaceutical Education & Research (NIPER), India. All the animal studies protocols were duly approved by the Institutional Animal Ethics Committee (IAEC), NIPER, India. Throughout the study, the animals were housed in laminar flow, at temperature of 25 ± 2 °C and relative humidity of 50–60% under a 12 h light/dark conditions. Animals were divided into three groups. Group I received P-ENX equivalent to 4 mg/kg of ENX subcutaneously while group II received a dose of 4 mg/kg of ENX. Group III was kept as a control receiving normal saline. The animals were fasted for 12 h before administration of drug and conjugate. The blood samples were collected from retro-orbital Plexus puncture in tubes containing 0.11 M sodium citrate (blood: citrate, 9:1) at different time points and blood samples were centrifuged at 2500*g* for 15 min to separate the plasma. The ENX concentration in the plasma was quantified by anti-Xa chromogenic assay as described later in Section 3.7. The peak plasma concentration (C_{max}) and the time to reach maximum peak concentration (T_{max}) were obtained from the time vs. plasma concentration profile. The standard non-compartmental analysis was performed for estimation of absorption profile (Pharsight WinNonlin Professional version 5.1). AUC was estimated by the linear trapezoidal rule method. Paired *t*-test was performed for comparing the results using GraphPad InStat (Version 3.05).

3.7. Anti-Xa activity method

There are no reported methods for direct quantitative estimation of ENX in blood samples other than the anti-Xa assay method which measures anti-Xa activity of heparin and low molecular weight heparins like ENX [17]. Anti-Xa activity of ENX in the blood samples was measured using Technochrom anti-Xa assay kit (Technoclone GmbH, Austria). A calibration curve was first developed employing the anti-Xa kit prior to conducting the assay. The reagents were prepared as per label instructions. The standard solutions of different concentrations of ENX were prepared by dilution with plasma. The solutions were further diluted with reagent 1 (Tris EDTA buffer) in a 9:1 ratio (reagent 1: standard solution). All the reagents and samples were preheated to 37 °C before performing experiments. Firstly, 50 μl of diluted sample was taken in a 96 well plate and 50 μl of reagent 2 (Bovine Factor Xa) was added to it, followed by mixing and incubating for 60 s at 37 °C, then 50 μl of reagent 3 (chromogenic substrate) was added, mixed and incubated for 30 s at 37 °C. Finally, 50 μl of 2% citric acid solution was added to stop the reaction, mixed thoroughly and the absorbance was measured at 405 nm by a MultiSkan Thermo 96-well plate reader. A blank sample was prepared by mixing the reagents in reverse order from that of the test procedure. Calibration curve was obtained from a plot of absorbance vs. standard plasma concentration.

4. Conclusion

In order to develop a long-acting formulation of ENX, PEGylation approach was employed. P-ENX was synthesized by conjugation of ENX with mPEG2000 via an ester linkage employing carbodiimide chemistry, using EDAC as coupling agent and DMAP as a base catalyst. The conjugate was purified by dialysis membrane (MWCO 2000). FTIR, ^{1}H NMR and MALDI analytical techniques were employed for its characterization. FTIR analysis provided direct evidence to P-ENX formation by revealing an increased stretching frequency of carbonyl group indicating the formation of an ester linkage between ENX and mPEG2000. However, ^{1}H NMR spectrum of the conjugate did show changes in the values of the chemical shift indicated interaction of ENX and mPEG2000 but was not conclusive. MALDI mass analysis displayed small peaks at 12,907 and 16,137 *m/z* confirmed the probability of ENX conjugation with mPEG 2000. It appears that on an average, approximately four to six molecules of mPEG2000 have been attached to a single molecule of ENX. The anti-Xa activity of the P-ENX conjugate as compared to the drug, as a measure of its *in vivo* bioavailability, showed a considerable increase in half-life (by about three-folds). Similarly, an increase in the AUC of P-ENX conjugate (four folds – 147.65 vs. 35.63 $\mu g\, ml^{-1}\, h^{-1}$) in comparison to the pure ENX confirms P-ENX's increased therapeutic potential. Thus, we can conclude that PEGylation of ENX can be adopted for enhancement of its half life in order to develop a long-acting formulation of ENX with a potential of decreased dosing frequency.

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