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Preparation and evaluation a new generation of low molecular weight heparin

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ABSTRACT

Enoxaparin is widely used in clinic, but it has some disadvantages. For example, its anticoagulant activity is weaker compared with heparin and it can not be effectively neutralizad by protamine sulfate (PS) in case of bleeding. Therefore, in this work, a new generation of low molecular weight heparin (NG-LMWH) was prepared.The NG-LMWH was prepared with the method of alkaline β-elimination followed by gel chromatography. Estimating the molecular weight of the NG-LMWH by GPC-HPLC, it has a remarkably low polydispersity index and narrow molecular weight distribution. The polydispersity index of NG-LMWH was 1.052, which was lower than heparin (1.5) and enoxaparin (1.279). Anti-FX_a and anti-FII_a potency of NG-LMWH was much higher than that of Enoxaparin, and close to that of heparin, which was determined by chromogenic substrate method. To test the degree of anti-FX_a or anti-FII_a potency neutralized by PS, equivalent anti-FX_a or anti-FII_a activity doses of different anticoagulant in plasma were titrated with increasing amounts of PS in plasma. The results indicate that NG-LMWH was more efficiently neutralized by PS than enoxaparin.The efficacy of anti-thrombus of NG-LMWH was superior to enoxaparin and the effect was dose dependent, which was evaluated with rat carotid artery thrombosis and inferior vena cava thrombosis model. The results of pharmacokinetics in New Zealand rabbits showed that the pharmacokinetic characteristics of NG-LMWH were similar to enoxaparin. The NG-LMWH prepared in this work has both advantages of heparin and enoxaparin with more effective and safer anticoagulation than enoxaparin.

1. Introduction

As the first line anticoagulant, heparin has been widely used for prevention of thrombosis in clinic for nearly 80 years. Its anticoagulant mechanism has been clarified. Heparin binds to the serine protease inhibitor antithrombin (AT) [1], causing it to undergo a conformational change resulting in AT strong inhibition of thrombin factor II_a and factor X_a [2]. But heparin is a heterogeneous mixture of polysaccharide molecules with large polydispersity in length of polysaccharide chain resulting in certain shortcomings, for examples, frequent bleeding [3], less predictable pharmacokinetic characteristics, big individual differences [4]. But protamine sulfate, as approved antidote of heparin, can effectively neutralize the anticoagulant activity of heparin in case of bleeding [5].

Low molecular weight heparins (LMWHs) present in 1980s were obtained by depolymerization of unfractionated heparin with physic-chemical methods or enzymatic methods [6]. Now they have taken over the majority application of heparin in clinic. The length of polysaccharide chain of LMWHs is generally less than eighteen monosaccharides, so the AT-mediated anti-FII_a activity of the LMWH is weakened [7]. The antithrombotic activity of LMWH is based on the AT-mediated anti-FII_a and anti-FX_a. Therefore, the anticoagulant activity and antithrombotic activity of LMWH are lower than heparin. However, LMWH can reduce nonspecific binding to endothelium and plasma proteins and the incidence of heparin-induced thrombocytopenia [8,9]. Besides, LMWHs have a longer half-life, a more predictable dose response [10], and a higher bioavailability. Although less bleeding frequency of LMWHs, once bleeding occurs, the anticoagulant activity of LMWHs can not be effectively neutralized by PS [11]. This is the partially risk of LMWHs in clinical application.

Therefore, heparin and the existing LMWH have their own advantages and disadvantages in clinic. It has been confirmed that the activity and pharmacokinetic characteristics of heparin and LMWHs are dependent on their molecular mass and distribution. In this work, by controlling the length and polydispersity of polysaccharide chains of fragmented heparin, a rational designed

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new generation of LMWH (NG-LMWH) is prepared. The anticoagulant activity of NG-LMWH was as strong as heparin, and it can be effectively neutralized by PS. Similar to LMWH, the pharmacokinetic characteristics of NG-LMWH was predictable.

2. Materials and Methods

2.1. Materials and animals

Heparin sodium and enoxaparin sodium were obtained from Dongying Tiandong Biochemical Industry Co., Ltd (Dongying, China); sodium citrate was from Sigma; S-2238 (*H-D*-Phe-Pip-Arg-pNA·2HCl), S-2765 (*N*-α-*Z-D*-Arg-pNA·2HCl), factor X_a, antithrombin III was from Chromogenix (Italy); protamine sulfate and human thrombin were obtained from National Institutes for Food and Drug Control (China); Activated partial thromboplastin time kit and prothrombin time kit were purchased from Sunbio Co., Ltd (Shanghai, China). All other chemicals were analytical reagent grade from China.

Male New Zealand rabbits weighing 2.0–2.5 kg were purchased from Jinan Xilingjiao experiment animal breed factory; male wistar rats weighing 200–250 g were obtained from the Laboratory Animal Center of Shandong University. All experimental procedures performed were approved by the Institutional Animal Care and Use Committee of National Institute Pharmaceutical Education and Research.

2.2. Preparation and purification the β-elimination LMWH

Through control of β-elimination conditions as described[12], the β-elimination LMWH was prepared. The obtained β-elimination LMWH was applied to a Bio-gel P10 column with a sample injection volume of 1 ml (100 mg), at the flow rate of 0.2 ml/min. The mobile phase consisted of 0.1 M NaCl and the detection wavelength was 234 nm. Fractions of 1.5 ml were collected in tubes.

2.3. Harvesting the targeted molecular weight range fractions

The molecular weights of samples harvested last step in fraction collector tubes were determined by GPC-HPLC method with reference substance. TSK-GEL G2000 SW_{XL} (30 cm) size exclusion column was used at a flow rate of 0.6 ml/min. The mobile phase was 0.2 M Na_2SO_4 (pH 5.0), and the detection wavelength was 232 nm and 215 nm. A sample injection volume was 100 μl. The average molecular weight of the samples in each tube was obtained. Combine the fractions with molecular weight distribution between 5400 Da and 9000 Da.

2.4. Estimating the molecular weight

GPC-HPLC was used to determine the molecular weight of the mixed sample and the sample injection volume was 10 μl (100 mg/ml). The experimental procedure was the same as mentioned above.

2.5. Desalination and desiccation of the samples

The samples were applied to a Sephadex G–10 column (100 × 1 cm) with a sample injection volume of 10 ml. The polysaccharides were eluted by pure water at a flow rate of 2.0 ml/min. After desalination, the sample was evaporated and concentrated, then freeze drying. The NG-LMWH was thus obtained.

2.6. Determination of the anticoagulant activity of the NG-LMWH

The anti factor X_a and anti factor II_a activity of the NG-LMWH were determined with the method of chromogenic substrate. The detailed procedure was described in the reference [5,13]. The S-2765 was used as the chromogenic substrate for anti-FX_a assay. The results were expressed in anti-FX_a IU/mg. The anti-FII_a was done similarly, but the thrombin-specific substrate S-2238 was substituted.

2.7. Evaluation the anticoagulant activity of the NG-LMWH neutralized by PS in vitro

The chromogenic substrate method was used. In briefly, heparin, NG-LMWH and enoxaparin were dissolved in deionized water to a final concentration of 0.1 mg/mL. Add heparin, enoxaparin and NG-LMWH solutions to a 48-well plate, respectively, then add plasma and the antidote (PS) [5]. Controlling the ratio of the PS to the samples was 0:1, 1:4, 1:2, 1:1, 3:2, 2:1, 3:1, respectively. In order to achieve the same total volume supply the saline. The 48-well plate was incubated at 37 °C for 20 min. The absorbance can be measured by UV-spectrophotometer. According to the drawing standard curve and linear regression equation, calculate the anti-FII_a activity of the samples after neutralized by PS. Evaluating the anti-FX_a activity of the NG-LMWH after neutralized by PS was the same as above mentioned.

2.8. Evaluation the antithrombotic activity of NG-LMWH in vivo

Forty-eight 8-week-old healthy Wistar rats, half male and half female, 200–250 g, were randomly divided into six groups: Saline group (s.c.), Heparin group (i.v., 3 mg/Kg), Enoxaparin group (s.c., 3 mg/Kg), NG-LMWH group (s.c., 1 mg/Kg), NG-LMWH group (s.c., 3 mg/Kg), NG-LMWH group (s.c., 5 mg/Kg). Before the experiment, Wistar rats were anaesthetized by intraperitoneal injection of 50 mg/kg body weight of pentobarbital sodium.

2.8.1. Carotid artery thrombosis

The antithrombotic activity of heparin, NG-LMWH and enoxaparin were investigated in rats with carotid artery thrombosis [5]. Briefly, under anesthesia, the carotid artery were exposed, and the filter paper (0.5 cm x 1 cm) saturated with 10% $FeCl_3$ was applied to the adventitial surface of the carotid artery. After 30 min, the filter paper was removed from the carotid artery. Firstly, weighed the total weight of 1 cm of carotid artery, then weighed the vessel which was removed the thrombus. The thrombus wet weight is the result of the subtraction of the two mixed with sodium citrate (9:1, v/v). Acquire the plasma samples by centrifugation with 3000r/min for 15 min. Citrated plasma samples were used in the APTT assay [14] and PT assay.

2.8.2. Inferior vena cava thrombosis

The effect of heparin, NG-LMWH and enoxaparin on vein thrombosis were investigated inferior vena cava thrombosis model [15,16]. The abdomen of each animal was opened and the vena cava was carefully dissected. A segment of 1.5 cm was prepared beginning just below the branch of the right renal vein up to after the left renal vein, which was ligated. In order to prevent peritoneal adhesion, 2 ml warm saline was injected into the abdominal cavity. All animals were administered with human thrombin (50IU/Kg) by tail vein injection. After 1 min, use operation line to ligate the vein, causing infraction. After 30 min, cut this vein and put it in the culture plate with 3.8% sodium citrate. The degree of thrombosis was evaluated by the Wessler method scoring criteria showed in Table 1 [16]. The thrombus wet weight was weighed immediately after water absorption by filter paper.