

Original Article

NOVEL SUBCUTANEOUS SUSTAINED RELEASE NANOPARTICLES ENCAPSULATING LOW MOLECULAR WEIGHT HEPARIN (LMWH): PREPARATION, CHARACTERIZATION AND EVALUATION

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ABSTRACT

Objective: The objective of the current research work was to prepare and evaluate novel subcutaneous sustained release polymeric nanoparticles for low molecular weight heparin (LMWH).

Methods: In this study, we prepared subcutaneously administered polymeric nanoparticles encapsulating LMWH using different grades of polycaprolactons (PCL) (14k, 45k, 80k) and 0.1% Polyvinyl alcohol (PVA) solution as surfactant by employing water-in-oil in-water (w/o/w) emulsion and evaporation method. The formulated nanoparticles were evaluated for size, shape, zeta potential, *in vitro* drug release, and *in vivo* biological activity (anti factor Xa activity) using standard kit, antithrombotic activity in thrombosis induced rat model. Drug and polymer interactions in the nanoparticles were evaluated using Fourier Transform Infrared Spectroscopy (FTIR), X-ray Diffraction (XRD).

Results: Scanning electron microscopic (SEM) studies on the nanoparticles confirmed the formation of spherical particles with smooth surface. The size of the formed nanoparticles were about 415-495 nm. The % entrapment of nanoparticles was found to be between 69-81%. Nanoparticles showed slow and sustained pattern of release for about 59-65 % in 48 h. Optimized nanoparticles exhibited excellent improvement in pharmacokinetic parameters and showed good antithrombotic activity, Activated partial thromboplastin time (aPTT) activity when compared to free drug. FTIR studies indicated that there was no loss in chemical integrity of the drug upon fabrication into nanoparticles. XRD results demonstrated that the drug changed its physical form in the formulation.

Conclusion: The results of this study revealed that subcutaneous nanoparticles were excellent candidates for sustained drug delivery of LMWH to avoid repeated subcutaneous administration.

Keywords: Low molecular weight heparin, Subcutaneous, Stability, Polycaprolactone, Venous thrombosis, Activated partial thromboplastin time

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INTRODUCTION

Low molecular weight heparin (LMWH) is an anticoagulant used in the prevention and therapy of venous thrombosis (VT). LMWH poorly absorbed in the gastrointestinal tract because of its large size, anionic charge, and enzymatic degradation and first pass metabolism [1,2]. So, it has to be administered via the parenteral (intravenous/ subcutaneous) route. LMWH requires repeated subcutaneous administration in 24 h duration, which is a major drawback in clinics [3]. Recently, various studies addressed subcutaneously administered sustained release LMWH nanoparticles using PCL, and these studies demonstrated sustained release of drug for 2-10 d *in vitro* and *in vivo* [4, 5].

Polycaprolactone (PCL) is a biodegradable, hydrophobic polymer approved by the Food and Drug Administration (FDA) for a variety of purposes [6]. It has been used in the development of various control release particulate delivery systems, due to its ease of preparation, compatible with numerous other polymers, versatility, biocompatibility, commercial availability at reasonable cost. Further, it is a hydrophobic polymer and upon hydrolytic degradation produces harmless and reasonable products [6, 7]. In the present investigation, we chose three different molecular weight polycaprolactones to prepare novel LMWH nanoparticulate control release delivery systems for subcutaneous administration to prolong the release of drug in the body to avoid repeated administration. Nanoparticles are popular dosage forms by now used in a variety of therapeutic applications [8, 9].

Polycaprolactone-based control release systems were recently addressed. Yerragunta et. al., developed a 3-month drug releasing

risperidone microspheres using blends of PCL. In this study, the developed formulation exhibited 90 d sustained drug release *in vitro* and *in vivo*. Furthermore, optimized formulation pharmacodynamics of the drug was measured using locomotor testing, locomotor activity of the optimized formulation was reduced during the study period when compared to the control [6]. Navitha et. al., recently developed 3 mo PCL based drugs-releasing implant for risperidone. In this study, a 12 w steadily sustained release risperidone PCL microsphere without a lag period was investigated using different molecular weight PCLs. Optimized formulation exhibited sustained zero-order release behaviour for 90 d without initial burst release and lag phase [7].

Some attempts to develop effective subcutaneous sustained release dosage forms for LMWH have been reported. Pazzini et. al., [2015] developed subcutaneous polymeric nanoparticles encapsulating enoxaparin using PCL and tested in venous thrombosis rat model. This study exhibited good results in relation to the encapsulation efficiency and shown sustained release of drug for a greater period time about 16 h *in vivo* than that of free drug solution [5]. Jogala et. al., [2015] developed subcutaneous sustained release nanoparticles for LMWH using PLGA (50:50,85:15) and tested in an animal model. This study demonstrated good encapsulation efficiency and sustained drug release *in vitro* and *in vivo* for 10 d. Furthermore, optimized formulation exhibited a four-fold enhancement in AUC_{0-∞} compared to free enoxaparin [4]. Choubey et. al., developed pegylated enoxaparin (P-ENX) for subcutaneous sustained release. This study exhibited excellent results in relation to the pharmacokinetic parameters. P-ENX (pegylated enoxaparin) has shown about three-fold enhancement in half life and four-fold enhancement in AUC in comparison to the pure enoxaparin [10].

In this study, our goal was to develop novel subcutaneous sustained release nanoparticles for LMWH and to evaluate various *in vitro*, *in vivo* parameters such as pharmacokinetic parameters, activated partial thromboplastin time, thrombotic activity in venous thrombosis rat model. Further, the drug properties in optimized formulations were determined and the results are discussed

MATERIALS AND METHODS

Materials

LMWH (Enoxaparin) was a gift sample from Gland Pharma Pvt. Ltd. (Hyderabad, India). PCL (14k45k, 80k) were purchased from Sigma-Aldrich Ltd., Germany. Dichloromethane and polyvinyl alcohol were purchased from SD Fine chemicals Ltd. A UV-Visible spectrophotometer from Thermo scientific was used. Probe sonicator (Qsonica, USA), Cooling centrifuge (Hittech, MIKRO 220R, Germany), Freeze dryer, Mini Lyodel (Delvac pumps, Chennai India) were used for the formulation of Nanoparticles. A JSM-5200 Scanning Electron microscope (SEM) Japan, was used to study the surface morphology of Nanoparticles. Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) was used to measure the particle size and zeta potential of prepared nanoparticles. Fourier transforms infrared spectrophotometer (FTIR) from Perkin-Elmer and X-ray diffractometer (XRD) from PAN Analytical were used. All other ingredients used in this study were of analytical grade

Methods

Preparation of nanoparticles

The LMWH nanoparticles were prepared by the water-in-oil in-water (w/o/w) emulsion and evaporation method. The required quantity of drug was taken in 2 ml of PVA solution (1%). To this 10 ml of methylene chloride containing the polymer (PCL 14000, PCL 45000, PCL 80000) was added and emulsified with a probe sonicator (Sonica) for 30 s at 60W. The resulting w/o emulsion was then poured into 40 ml of PVA (0.1%) solution and sonicated for 60 s at 60W using probe sonicator involving in the formation of w/o/w emulsion. This emulsion was kept on a magnetic stirrer (15 rpm) for 10 min to evaporate methylene chloride. Nanoparticles were then isolated by centrifugation (Hittech, MIKRO 220R), at 18000 rpm for 15 min.[11,12] The supernatant was removed and assayed to find out entrapment efficiency. The nanoparticles were washed three times with deionized water and subjected to freeze drying (Lyophilizer, MINI-LYODELDELVAC, Chennai, India).

Size and surface analysis

The mean diameter of nanoparticles and their surface potential were evaluated with Zeta sizer. Surface morphology was evaluated using scanning electron microscopy (SEM).

Scanning electron microscopy

In order to examine the particle surface morphology and shape, scanning electron microscopy (SEM) was used. A concentrated aqueous suspension was spread over a slab and dried under vacuum. The sample was shadowed in a cathodic evaporator with gold layer 20 nm thick. Photographs were taken using a JSM-5200 Scanning Electron Microscope (Tokyo, Japan) operated at 20 KV. The smallest size nanosuspension was used for determining surface morphology.

Zeta potential

The zeta potential is used to measure the electric charge on the surface of the particles, indicating the physical stability of colloidal systems. In this study, the zeta potential was assessed by determining the electrophoretic mobility of the particles. The zeta potential was measured using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Samples were diluted with the respective original dispersion medium which provides information regarding the thickness of the diffuse layer. Diluted nanosuspension was added to the sample cell (quartz cuvette) and was put into the sample holder unit, and zeta potential was measured.

Encapsulation efficiency

The amount of drug entrapped in nanoparticles was determined by turbidimetric assay by measuring the amount of non-entrapped

drug in the supernatant recovered after centrifugation. Briefly, 1 ml of supernatant was taken to this 1 ml of acetate buffer (1M, pH 5) followed by 4 ml of cetyl pyridinium solution (0.1%) in NaCl 0.94% and assayed for drug release at 500 nm by UV spectrophotometer [13].

In vitro drug release

An aliquot of 10 mg of nanoparticles was suspended in a flask containing 25 ml of phosphate buffer saline (PBS 0.011M, NaCl 0.15M, pH7.4), and kept it for stirring (150 rpm) on a magnetic stirrer. 1 ml of sample was taken at 1,3,6,9,12,18,24,30,36 and 48 h using a syringe filter. To this 1 ml of acetate buffer (1m pH 5) followed by 4 ml of cetyl pyridinium solution (0.1%) in NaCl 0.94% were added and the drug was assayed at 500 nm by UV spectrophotometer [11, 13].

Selection and characterization of optimized formulation

Using size, zeta potential, encapsulation, *in vitro* drug release, the optimum formulation was selected. The optimized formulation was characterized for various properties.

In vivo drug release from LMWH nanoparticles

The *in vivo* biological activity of LMWH was evaluated by measuring the anti-factor Xa activity with a chromogenic substrate by using a standard kit (KRIBIOLISA™ Xa) from Krishgen Biosystems according to the method described by the supplier. *In vivo* biological activity of LMWH was investigated in male Wistar rats. All the experiments were conducted according to the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision on Experimental Animals). The study was approved by an animal ethical committee of Synapse Life Sciences; Warangal registered under CPCSEA, India. The animals were divided into 2 groups(n=6). The groups are as below:

Group 1: Subcutaneous administration of PNP2 formulation

Group 2: Subcutaneous administration of LMWH solution

Group 1 received 0.3 ml suspension of PNP2 formulation subcutaneously. Group 2 received 0.3 ml suspension LMWH solution. All the formulations administered had equal amounts of LMWH. After administration of formulations blood samples were collected at 6, 12,18, 24 h and 2,3,4,5,6,7,8,9, and 10 d and the samples were subjected to centrifugation for 10 min at 5000g, using centrifuge (REMI RM 12C), then the plasma collected and anti-factor Xa activity was measured using a standard kit (KRIBIOLISA™ Xa) from Krishgen Biosystems according to the method described by the supplier [4, 14, 15].

Antithrombotic activity in animal model of venous thrombosis

The rats were divided into 3 groups (n=6) with an average weight of the rats was 250g. All the rats were anesthetized by intraperitoneal injection of a solution of ketamine (0.15 ml), xylazine (0.5 ml) and deionized water (0.75 ml) in does a dose of 0.2 ml for 100 g of weight. From the *in vitro* evaluation, PNP2 formulation was selected as optimized formulation. A 4.5 mg/kg of PNP2 formulation was injected subcutaneously to one group; a second group was injected with 4.5 mg/kg LMWH solution, and the third group was injected with normal saline solution subcutaneously. One hour after the administration, the abdomen of the animals was surgically opened, and thrombosis was induced according to the modified method [11,16]. Three hours after thrombosis induction, animals were anaesthetised, and the abdomen reopened to verify the presence of thrombus. If thrombus was present, it was withdrawn and weighed immediately and after drying for 24 h at 37 °C.

Estimation of activated partial thromboplastin time (aPTT)

Blood coagulation-indicating parameter, namely activated partial thromboplastin time, was measured in male Wistar rats. All the experiments were conducted according to the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision on Experimental Animals). The study was approved by an animal ethical committee of Synapse Life Sciences; Warangal registered under CPCSEA, India. The animals were divided into 2 groups (n=5). The day before the experiments, all the rats were cannulated from

right jugular vein according to a standard procedure using a polyethylene catheter connected to a silicone rubber tubing. For complete recovery from anesthesia and surgery, the animals were then kept individually overnight in a temperature-controlled room with a 12-h light-dark daily cycle. During this period, the animals were kept in standard cages with free access to rat chow and water. On the day of experiments, group 1 and group 2 of the rats received a 200 IU/kg dose of free heparin and the equal dose of drug-loaded nanoparticles, respectively, via the catheter. At the time just before the drug administration (time 0), and also at 1,3,6,12,18,24,30,26,42, and 48 h following the heparin doses, 0.5-ml blood samples were obtained via the catheters and were mixed immediately with trisodium citrate 3.2% (0.109 M) with a volume ratio of 9: 1 (blood: sodium citrate) before centrifuging for 15 min at 1500 *g* to obtain plasma. 100 μ l of plasma was taken in a cuvette and pre-warmed to 37 °C. Then, 100 μ l of the aPTT-XL reagent was added onto the plasma, and the mixture was incubated for 4 min at 37 °C. At the end of 4 min, 100 ml of pre-warmed calcium chloride solution (0.02 M) was added to the plasma-reagent mixture, and clot formation was timed [11, 17].

Table 1: Composition of various polymeric nanoparticle (PNP) formulations

| Ingredients | PNP1 | PNP2 | PNP3 |
|--------------------------|------|------|------|
| LMWH (mg) | 20 | 20 | 20 |
| PCL 14000 (mg) | 100 | --- | --- |
| PCL 45000 (mg) | --- | 100 | --- |
| PCL 80000 (mg) | --- | --- | 100 |
| Dichloromethane (ml) | 10 | 10 | 10 |
| PVA solution (0.1%) (ml) | 40 | 40 | 40 |
| Water (ml) | 2 | 2 | 2 |

RESULTS AND DISCUSSION

Low molecular weight heparin is the drug of choice for various diseases where its anticoagulant activity useful. Further, it has an insignificant oral bioavailability, short half life and needs to repeat subcutaneous administration [11]. In the present investigation, for the first time, we fabricated polymeric subcutaneous sustained release nanoparticles encapsulating LMWH using polycaprolactones of different molecular weights. Nanoparticles were successfully prepared by water-in-oil-water (w/o/w) emulsion and evaporation method. Three formulations were prepared using different grades of PCL (14k, 45k, 80k) and 0.1% PVA as a surfactant.

Poly caprolactone (PCL) is a type of synthetic, FDA-approved biodegradable polymer; FDA approved applying in food packaging, tissue engineering, drug delivery, So it becomes one of the most promising biodegradable polymers currently available on the market. PCL have gained enormous prominence because of its practical and market viable drug delivery applications. It is also in the search for appropriate matrices for control drug delivery microspheres and nanoparticles, because of its commercial availability at reasonable cost, ease of preparation, biocompatibility, versatility and its hydrolytic degradation products are resorbable, harm-less [7]. However, some shortcomings such as low melting temperature and low mechanical properties restrict widespread use of PCL.

Recently Pazzini et. al., developed subcutaneous polymeric nanoparticles of enoxaparin using PCL and tested in venous thrombosis rat model. This study has shown good encapsulation efficiency (80%) and demonstrated sustained release of enoxaparin from encapsulated nanoparticles for a greater period of time (16 h) *in vivo* than that of free enoxaparin. Furthermore encapsulated enoxaparin nanoparticles demonstrated excellent antithrombotic activity in venous thrombosis rat model [5]. Recently Navitha et. al., and Yerragunta et. al., developed control drug release risperidone implants, microspheres respectively. Wang et. al., developed Disodium nor canthari date loaded PCL microspheres by W/O/W emulsion solvent evaporation, this study demonstrated good results in relation to particle size and encapsulation efficiency, initial burst release followed by sustained release of drug from microspheres [18]. Chawala et. al., developed PCL based tamoxifen nanoparticles for tumour-targeted delivery. This study demonstrated the formation of spherical shape and uniform size (250-300 nm)

Fourier transforms infrared spectroscopy (FTIR)

FTIR spectrum of drug, polymers, physical mixture and formulations were obtained on FTIR instrument. Sample about 2 mg was mixed thoroughly with 100 mg potassium bromide IR powder and compacted under vacuum at a pressure of about 12 Psi for 3 min. The resulting disc was mounted in a suitable holder in Shimadzu IR spectrophotometer, and the spectrum was scanned over the wave number range of 4000-400 cm^{-1} . IR helps to confirm the identity of the drug and to detect the interaction of the drug with the carriers.

Powder X-ray diffractometry (PXRD)

The XRD patterns of Drug, PEG, polymer, physical mixture and optimized formulation were obtained using X-ray diffractometer (Shimadzu 770). The measuring conditions were as follows: Cu K α , radiation, nickel filtered; graphite monochromator; 40 KV voltage; and 30 mA current with X'celerator detector. All samples were run at 1° (2 θ) min from 3° to 45° (2 θ).

nanoparticles. Furthermore, fabricated nanoparticles demonstrated good encapsulation efficiency (64%).

In the present study, the particle size of the all the nanoparticle formulations was found between 415-495 nm. The study of SEM was conducted to confirm the formation and surface morphology of nanoparticles. All the nanoparticles were spherical in shape with a smooth surface. These were shown in fig. 1. The nanoparticles exhibited negative surface charges; this negative charge increases the stability of prepared nanoparticles by minimizing the agglomeration of nanoparticles, the negative charge of the particles due to the presence of low molecular weight heparin, surfactant, and the polymers. Here PVA works as particle stabilizer and increases the stability of nanoparticles probably due to steric stabilization phenomena [5, 11]. The Encapsulation efficiency of the prepared particles was determined by turbidimetric assay method. Encapsulation efficiencies of the nanoparticles PNP1, PNP2, PNP3 are 71 \pm 0.31, 81 \pm 0.25, 69 \pm 0.25 respectively. The maximum encapsulation efficiency (81 \pm 0.25) was exhibited by PNP2 nanoparticles. PCL 25 k is a hydrophobic polymer, even though it exhibited good encapsulation efficiency, a recent study reported the similar results by using PCL 45K [5]. Thus, this method of preparation is suitable for encapsulation of hydrophilic drugs in polymeric nanoparticles. The results of particle size, encapsulation efficiency, zeta potential are depicted in table 2.

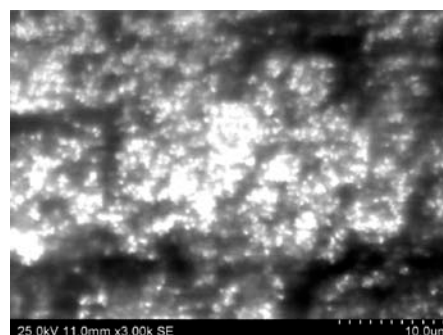


Fig. 1: Scanning electron microscope pictures of the nanoparticles

Table 2: Particle size, zeta potential and encapsulation efficiencies of all nanoparticle formulations

| Formulation | Particle size (nm) | Zeta potential (mv) | Encapsulation efficiency (%) |
|-------------|--------------------|---------------------|------------------------------|
| PNP1 | 415±1.21 | -17.4±0.8 | 71±0.31 |
| PNP2 | 425±2.12 | -23.5±0.6 | 81±0.25 |
| PNP3 | 495±3.35 | -26.1±0.7 | 69±0.25 |

The values indicates n=3, mean±SD

In vitro drug release of the, all the three formulations were performed in 25 ml of phosphate buffer saline (PBS 0.011M, NaCl 0.15M, P^H7.4) as per the method described above. The release profiles are shown in fig. 2. All the formulations sustained the release of the drug for a period of 48 h. PNP2 formulation exhibited slow and sustained release pattern and maximum drug release is about 65.25% in 48 h. This is must be due to the hydrophilic nature and its high crystallinity of the polymer (PCL). High crystallinity in the particles can lead to slow rate of drug release in the sustained pattern. From the results of *in vitro* evaluation of the nanoparticles, PNP2 formulation was selected and further subjected to *in vivo* characterization.

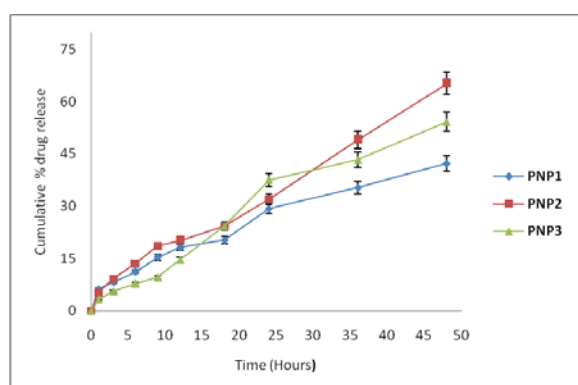


Fig. 2: *In vitro* drug release profiles of all the formulations (n=6, mean±SD)

In vivo drug release studies were performed for optimized (PNP2) formulation and pure drug solution. Plasma concentration vs time profile of the PNP2 formulation and pure drug solution was depicted in fig. 3. PNP2 nanoparticles and the low molecular weight heparin solution were administered subcutaneously to the rat, and the blood samples were withdrawn, and plasma was collected at different time intervals and then anti-factor x_a activity was measured PNP2 formulation exhibited excellent results when compared to the free drug solution. For pure drug solution peak plasma concentration (C_{max}) was 14.9 µg/ml, T_{max} was 1h, AUC_{0-∞} was 40.452 (µg/ml/h) and after 6 h concentration was 0.2 µg/ml. PNP2 formulation exhibited prolonged plasma concentration for 24 h. Peak plasma concentration (C_{max}) was 13.9 µg/ml, and, T_{max} was 12 h, AUC_{0-∞} was 141.25 (µg/ml/h) After 24 h concentration of drug in plasma was about 3.2 µg/ml. Optimized formulation exhibited 3.5 times enhancement (40.452 vs 141.25 µg/ml/h) in area under curve (AUC_{0-∞}). This is must be due to the hydrophobic nature of the polymer.

Antithrombotic activity of the optimized formulation (PNP2) and the pure drug solution, control (normal saline solution) determined in venous thrombosis induced rat model. The weight of the thrombi formed after subcutaneous administration of control, pure drug solution, and optimized formulation was to be 22.10±0.13, 4.4±0.21, 0.71±0.1 mg respectively.

The weight of the thrombi formed in the group of rats which received the optimized formulation was very less when compared to the weight of the thrombi formed in the groups of rats which received the pure drug solution and normal saline solution. This is probably due to the maintenance of the minimum of the concentration of the drug (0.26 µg/ml) in the plasma for a prolonged period of time which requires for the venous prevention thrombosis. The results were represented in fig. 4.

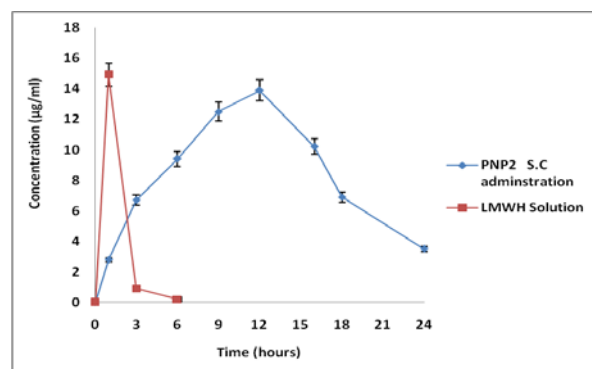


Fig. 3: Plasma concentration profiles of optimized formulation (PNP2) and LMWH solution after subcutaneous administration (n=6, mean±SD)

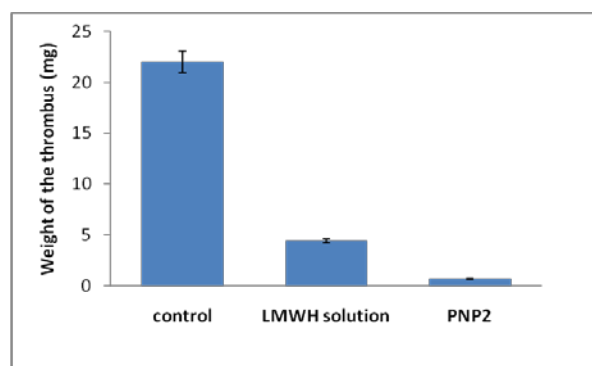
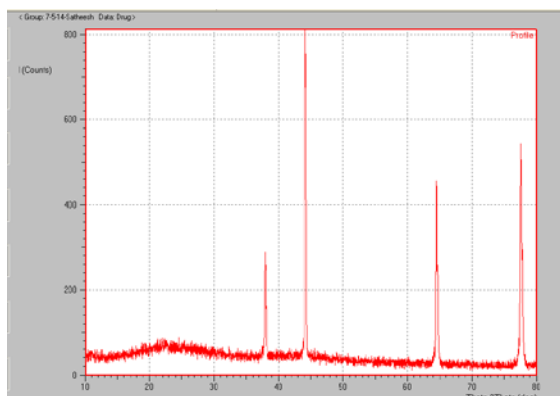


Fig. 4: Weight of thrombus after subcutaneous administration of control, free drug, and Optimized formulation (PNP2). (n=6, mean±SD)

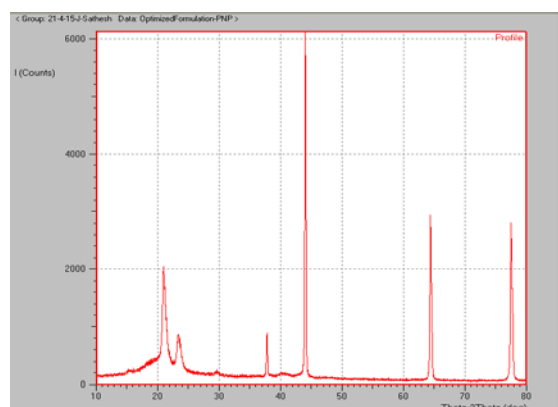
Activated partial thromboplastin time (aPTT) for pure drug solution and optimized (PNP2) formulation was estimated after subcutaneous administration. For free drug solution aPTT increased rapidly with average maximal aPTT of about 162 s occurred at 1 h. After subcutaneous of PNP2 formulation maximum aPTT of about 102 s occurred about in 18 h. aPTT of both free drug and optimized formulation increased from starting time of about 18 s to final value (162 s for a free drug, 102 s for optimized formulation). The free drug reached maximal aPTT very early, but the optimized formulation reached about in 10 h.

FTIR spectra of the drug, polymers and optimized formulation were recorded in the range of 4000-400 cm. The FTIR spectra of drug and PCL 45k compared with the FTIR spectra of the optimized formulation. The characteristic functional groups of drug and PCL 45k showed peaks in the following wavenumber. In pure drug there was C-O stretching at 1230 cm; N-H stretching at 3350 cm; N-H bending at 1624 cm. S=O peak at 1145 cm. In PCL 45K characteristic broad peak of O-H was there at 3500-3600 cm. C-H stretching sharp peak at 2850 cm. There was the appearance of same characteristic peaks are appeared in the optimized formulation this indicates there was no chemical interaction between drug and polymers used (data not shown). The presence of peaks in the expected range confirms that the materials taken for the

study are genuine, and there was no occurrence of possible interactions. XRPD study yielded interesting results. The drug was crystalline due to the sharp peaks demonstrated in XRPD. With the optimized nanoparticles, there was a small bump in the peak, suggesting partial conversion of LMWH to less crystalline form and that may be due to the formation of amorphous form upon formulation. XRPD spectras are shown in fig. 5.



A



B

Fig. 5: XRPD Patterns of LMWH (A: Pure Drug; B: Optimized Formulation)

CONCLUSION

In conclusion, in this investigation, we formulated novel and better subcutaneous sustained release nanoparticles for low molecular weight heparin (LMWH), optimized the formulation so as to demonstrate excellent results in relation to the particle size, encapsulation efficiency (81%) and *in vitro* sustained drug release for 48 h with about 65% drug release. Optimized formulation demonstrated 3.5-folds enhancement AUC when compared to a free drug solution. Furthermore, optimized formulation demonstrated excellent antithrombotic activity in venous thrombosis rat model. Based on the above results, these fabricated nanoparticles would serve as a promising prospect for the efficient prevention of venous thrombosis. The results were tempting for the clinical realization of the formulation and can be taken forward for further development.

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CONFLICT OF INTERESTS

The authors declare that we have no conflict of interest

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