



Development and Characterization of Chitosan-Alginate based Low Molecular Weight Heparin Novel Nanoparticles for Oral Delivery

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Abstract

Objective: The objective of the current research work was to prepare and evaluate novel chitosan-alginate based nanoparticles for oral delivery of low molecular weight heparin (LMWH). In this study, we prepared orally administered nanoparticles encapsulating LMWH using chitosan-alginate by employing Polyelectrolyte complexation 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. Scanning electron microscopic (SEM) studies on the nanoparticles confirmed the formation of spherical particles with smooth surface. The size of the formed nanoparticles was about 415-485nm. The % entrapment of nanoparticles was found to be between 46.5-80.5%. Optimized Nanoparticles formulation showed 93% drug release in 24 hrs. Optimized nanoparticles exhibited excellent improvement in pharmacokinetic parameters when compared to Clexane LMWH marketed product when administered orally. The results of this study revealed that oral nanoparticles are suitable candidates for targeted delivery of LMWH through colon route into systemic circulation.

Keywords

Low molecular weight heparin, Intravenous, Stability, Polycaprolactone, Venous thrombosis, activated partial thromboplastin time.

INTRODUCTION:

Low molecular weight heparins (LMWH) are one of the most potent anticoagulants in the prevention and the treatment of deep vein thrombosis and pulmonary embolism¹. Although various new pharmacological molecules have been developed, heparins remain the classical treatment in venous thromboembolism. However, the parenteral administration of LMWH by intravenous or

subcutaneous routes and repetitive injections are the main disadvantages.^{2, 3} Unfortunately, a major disadvantage of LMWHs is that they can only be administered parenterally, due to a lack of absorption when administered orally. Intestinal absorption of LMWHs is limited owing to their poor membrane permeability characteristics such as hydrophilicity, anionic surface charges, unstable in the acidic conditions of the stomach⁴. The

development of oral formulations for LMWH would have tremendous clinical importance as it would result not only in avoidance of the pain and discomfort associated with parenteral administration but would also reduce expenses associated with prolonged hospital stay and parenteral, thereby offering improved patient compliance⁵.

To overcome the poor oral bioavailability of LMWHs, various delivery approaches have been attempted including liposomes, microparticles, emulsions, enteric coating, complexation, and carrier systems.⁵⁻⁷ Unfortunately, no oral formulation is able to produce good oral bioavailability. Oral LMWH formulation is not available in the market yet now. Since LMWH is permeable in the lower small intestine and colon, its penetration enhancement can be conveniently used to produce a market viable oral formulation for LMWH.

Chitosan, a polycation with an apparent pKa 6.1-7.3 at acidic pH, is a plentiful natural polysaccharide found in Crustacean and obtained from N-deacetylation of chitin. Chitosan presents numerous benefits in terms of oral colon drug delivery, including being biodegradable, biocompatible, non-toxic, non-immunogenic, and colonically digested. In gentle situation chitosan can also form nanoparticles with polyphosphate through ionotropic gelation for protein and peptide drug loading.⁸ Chitosan forms a strong bond with the negatively charged epithelial lining of gastrointestinal tract. This high interaction helps in opening the tight junction and makes the drug reach systemic circulation. However, it is mostly soluble in acidic pH conditions of the stomach, which makes it to lose its mucoadhesive and permeation enhancing properties.^{9,10}

Sodium alginate is an anionic polysaccharide distributed widely in the cell walls of brown algae. It is a linear copolymer with homopolymeric blocks of (1-4)-linked β -D- mannuronate (M) and its C-5 epimer α -L-guluronate (G) residues, covalently linked together in different sequences or blocks. The negatively charged carboxylic acid groups of mannuronic and guluronic acid units of alginate interact electrostatically with positively charged amino groups of chitosan to form polyelectrolyte.¹¹⁻¹³

In this study, our goal was to develop Chitosan-Alginate LMWH novel oral nanoparticles and to evaluate various *in vitro*, *in vivo* parameters. 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). Sodium Alginate, Acetic acid was purchased from SD Fine Chemicals Limited, Gujarat, India. Chitosan, sodium tripolyphosphate (STPP), dialysis membrane (MW 12,000 Daltons), and cetyl pyridinium chloride were purchased from Sigma-Aldrich Private Limited, Mumbai, India. Simulated gastric fluid pH 1.2, simulated intestinal fluid of pH 6.8 and 7.4 were prepared by referring to official methods as specified in USP (XXV). Clexane (Enoxaparin Marketed Product) manufactured by Sanofi India Ltd. UV-Visible spectrophotometer from Thermo scientific was used, Cooling centrifuge (Hittech, MIKRO 220R, Germany), 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 Perkinelmer. All other ingredients used in this study were of analytical grade.

METHODS:

Preparation of Chitosan nanoparticles:

Nanoparticles were prepared by ion gelation of Chitosan with sodium tripolyphosphate (STPP) aqueous solution. First, Chitosan was dissolved in 1% solution of acetic acid. LMWH was then added to Chitosan solution and mixed. The aqueous solution of STPP was added drop wise to Chitosan solution by magnetic stirring at room temperature using high-speed homogenizer¹⁵. Nanoparticles were then isolated by centrifugation (Hittich, MIKRO 220R), at 18000 rpm for 15 min. The supernatant was removed and assayed to find out entrapment efficiency¹⁴.

Preparation of Chitosan-Alginate LMWH Nanoparticles:

Chitosan- Alginate LMWH nanoparticles were prepared by Polyelectrolyte Complexation method. Sodium alginate solution was prepared by dissolving Sodium alginate in water. Chitosan solution was prepared by dissolving CH in 1% acetic acid solution. Both the solutions were placed separately on magnetic stirrer at 100 rpm for 1 hr. LMWH was then added to Sodium alginate solution and dissolved. Sodium alginate solution was adjusted to pH 6.5 and Chitosan solution to pH 4.0. Sodium alginate solution is then slowly added to CH solution at a flow rate of 1 ml/s and then homogenized the solution at 10000rpm with the help of high-speed homogenizer¹⁴. Thus Alginate-chitosan LMWH

nanoparticles were formed. Nanoparticles were then isolated by centrifugation (Hettich, MIKRO 220R), at 18000 rpm for 15 min. The supernatant was removed and assayed to find out entrapment efficiency¹⁴.

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:

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 a vacuum. The sample was shadowed in a cathodic evaporator with a 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 at 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). Sample suspension 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 1ml of acetate buffer (1M, pH 5) followed by 4 ml of cetylpyridinium solution (0.1%) in NaCl 0.94% and assayed for drug release at 500nm by UV spectrophotometer¹¹.

In-vitro drug release:

The in vitro release study of LMWH from drug loaded PEC nanoparticles was performed using the dialysis membrane method. LMWH PEC nanoparticulate suspension was filled in a dialysis bag (MW-12,000 Da) which was attached to a two-end opened boiling tube^{8,9}. The boiling tube was then dipped in a beaker containing 50 ml of pH 1.2 buffer and placed on a magnetic stirrer for 2 hrs at 37±0.5°C and 50 rpm. The medium was then replaced with pH6.8 buffer and after 3 hrs with pH7.4 buffer. The release study was performed for 24 hrs. Aliquots of 0.5 ml were taken at regular intervals, i.e., 1, 2, 3, 4, 5, 6, 8, 10 up to 24 hrs, to this 1 ml of acetate buffer (1m pH 5) followed by 4 ml of cetylpyridinium solution (0.1%) in

NaCl 0.94% were added and the drug was assayed at 500nm by UV spectrophotometer¹⁴.

Using size, zeta potential, encapsulation, *in vitro* drug release, F5, F6 formulations were selected for ex vivo drug release study.

Ex vivo drug release study:

Ex vivo drug release study the amount of LMWH transported across the intestinal barrier was measured. Small intestine of male Wistar rat was removed and rinsed with normal saline solution. LMWH loaded PEC nanoparticulate (F5 and F6) suspension was filled in the membrane, and both ends were tied with a thread separately and fixed to a stand. The membrane was then introduced into 50 ml of 6.8 pH buffer in a beaker. The beaker was then placed on a magnetic stirrer at 37°C and 100 rpm. Aliquots of 1 ml were taken at intervals of 5, 15, 30, 45, 60, 90, 120 minutes up to 8 hrs. The samples were then analyzed by UV-visible spectrophotometer at 500 nm by the addition of reagents and reacted for 1 hr. Permeation studies were performed in triplicate.¹⁹

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 standard kit (KRIBIOLISA™ Xa) from Krishgen Bio systems 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 animal ethical committee of Synapse Life Sciences, Warangal registered under CPCSEA, India. The animals were divided into 3 groups (n=6).

The groups are as below:

Group 1: Oral administration of F5 formulation
Group 2: Oral administration of F1 formulation
Group 3: Oral administration of Clexane (LMWH marketed product)

All the formulations administered had equal amounts of LMWH (50mg/kg). After administration of formulations blood samples were collected from retro-orbital plexus at 0, 1, 2, 3, 4, and 5 up to 8 hrs and the samples were subjected to centrifugation for 10 min at 5000g, using centrifuge (REMI RM 12C), then the plasma collected anti factor Xa activity was measured using standard kit (KRIBIOLISA™ Xa) from Krishgen Bio systems according to the method described by the supplier⁶.

Fourier transform 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 minutes. The resultant 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.

Differential Scanning calorimetry (DSC):

Differential Scanning calorimetry was used to determine drug excipient compatibility studies and used to observe more phase changes such as glass transition, crystallization, amorphous forms of drugs and polymers. The physical state of drugs and polymer was analyzed by Differential Scanning calorimeter (Schimadzu). Approximately 5 mg of sample was analyzed in an open aluminum pan and heated at scanning rate of 10°C/min between 0°C and 400°C. Magnesia was used as the standard reference material.

RESULTS AND DISCUSSIONS:

In this study, Chitosan-Alginate LMWH nanoparticles (PEC nanoparticles) and Chitosan -LMWH nanoparticles. Chitosan -Alginate LMWH nanoparticles were prepared by polyelectrolyte complexation method and chitosan -LMWH nanoparticles were prepared by ion-gelation method. The prepared nanoparticles were evaluated for particle size, zeta potential, surface morphology, in vitro drug release and *in vivo* anti factor xa activity.

Determining the levels of ant factor Xa activity in plasma is an indication of drug release from the formulations. This is a routine assay used for determination of heparins in the plasma after various administration modes.

Drug and Excipient compatibility studies were conducted using FTIR and DSC. To formulate oral LMWH nanoparticle Sodium alginate and chitosan. The FTIR spectra of drug chitosan and Sodium alginate polymer was compared with the FTIR spectra of optimized formulation. FTIR results suggested that nanoparticles were formed and there was no chemical interaction between drug and polymers used. The presence of peaks at the expected range confirmed that the materials taken for the study are genuine and there were no possible interactions that occurred.

All the fabricated nanoparticle formulations were evaluated for particle size, zeta potential, encapsulation efficiency and the results were represented in Table.2. The particle size of all the formulations was found between 415- 485nm. All the Chitosan -Alginate LMWH nanoparticles (PEC nanoparticles) nanoparticles have good % entrapment efficiencies (46.5-80.5%). As the concentration of CH increases encapsulation efficiency was increase, this was because of, as the concentration of chitosan increased the number of complexes that are formed between chitosan and alginate were increased and other reason was the interaction between negatively charged LMWH and Chitosan. Nanoparticles formulations exhibited negative surface charges of the results were represented in Table.2.

Table 1: Composition of LMWH Nanoparticles

Formulation	F1	F2	F3	F4	F5	F6
LMWH (mg)	20	20	20	20	20	20
Chitosan (mg)	100	50	100	100	150	150
Sodium Alginate (mg)	--	50	50	125	75	125
Acetic acid (V/V)	1	1	1	1	1	1
Water (ml)	50	50	50	50	50	50

Table 2. Particle size, zeta potential and Encapsulation efficiencies of all nanoparticle formulations

Formulation	Particle Size (nm)	Zeta Potential (mv)	Encapsulation efficiency (%)
F1	485±1.12	-16.4±0.3	46.5 ±0.8
F2	415±2.12	-22.5±0.4	52.5 ±2.2
F3	476±3.32	-21.1±0.7	66.5 ±1.1
F4	462±2.21	-18.1±0.2	69.5 ±2.1
F5	451±2.31	-16.1±0.5	80.51±0.8
F6	451±1.23	-15.1±0.6	79.52 ±1.1

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. (Figure.1).

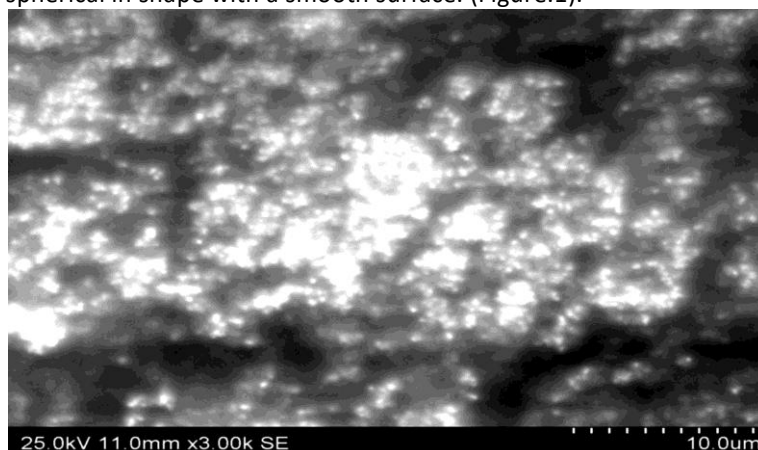


Figure 1. Scanning Electron Microscope pictures of the Nanoparticles

In vitro drug release of the, all the three formulations were performed by dialysis membrane method (pH 1.2 for 2h, pH 6.8h for 3h and in pH 7.4 buffer up to 24 hrs) as per the method described above. The release profiles are shown in Figure. 2. F2 -F6 formulations (Chitosan-alginate nanoparticles) released less than 1.5% of drug release in first 2 hrs in the pH 1.2 buffer, this is attributed to the insolubility of alginate in the pH. In pH 6.8 and pH 7.4 buffer constant releases LMWH was observed for all

the chitosan-alginate LMWH formulations, and the maximum drug release was about 88% within 10 hrs, this could be because of interaction of alginate with alkaline medium and increased the solubility of alginate. Plain chitosan LMWH nanoparticle formulation (F1) showed 29.5% drug release in pH 1.2 buffers. From the results of *in vitro* evaluation of the nanoparticles, F5 and F6 formulations were selected and further subjected to *in vivo* characterization.

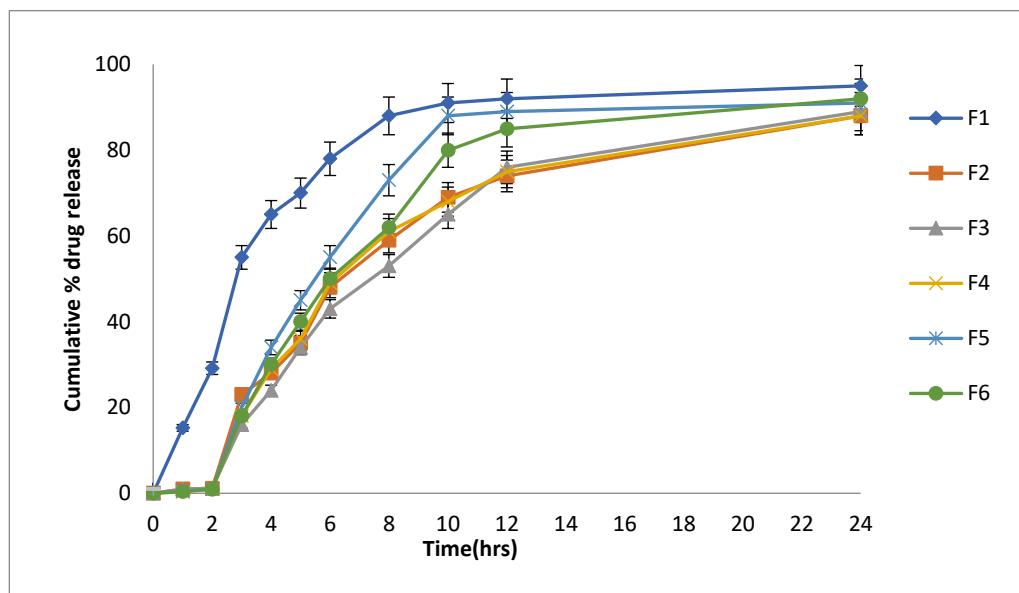


Figure 2. In-vitro drug release profiles of all the formulations.

Based on the data obtained from *in vitro* characterization, F5 and F6 formulations were selected, and this were further subjected to ex-vivo transportation study using small intestine of male wistar rats. In the results of ex vivo studies, from F5 formulation 72 % and from F6 formulation 59% of the drugs were found to cross the intestinal membrane

and reach the buffer in 6 hrs time period. The results were depicted in the Figure.3

In vivo evaluation in rats to estimate *in vivo* drug release by measuring anti factor X_a activity. Plasma concentration vs time profile of F1 formulation F5 formulation and marketed product Clexane are represented in Figure 4. Software was used to

calculate the pharmacokinetic parameters, and the results were represented in Table 3.

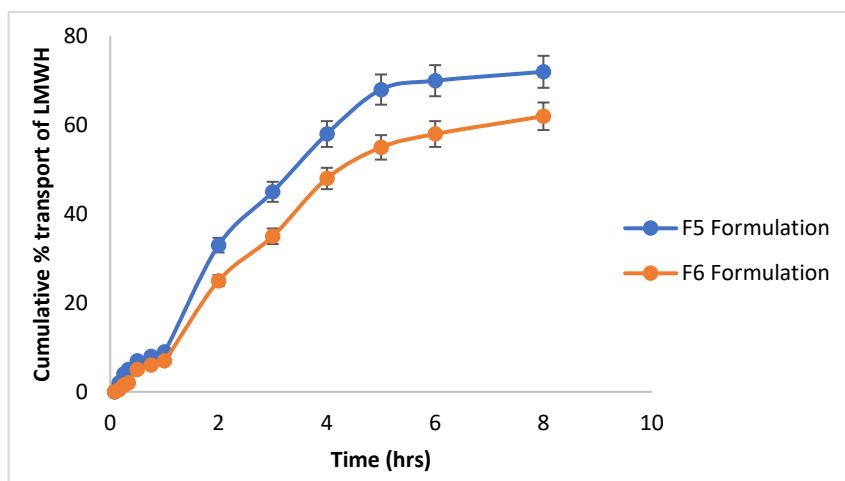


Figure 3. Cumulative percent transport of low molecular weight heparin Chitosan-Alginate LMWH nanoparticles (F5, F6).

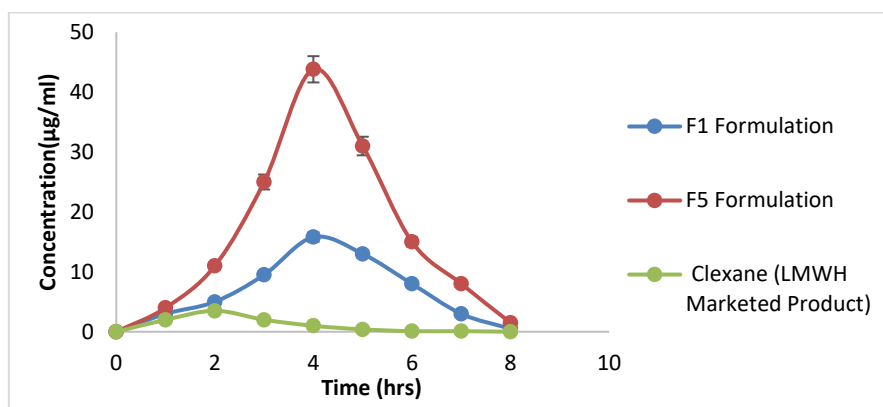


Figure 4. Plasma concentration profiles of F1 and F5 (Optimized) formulations and Clexane marketed product.

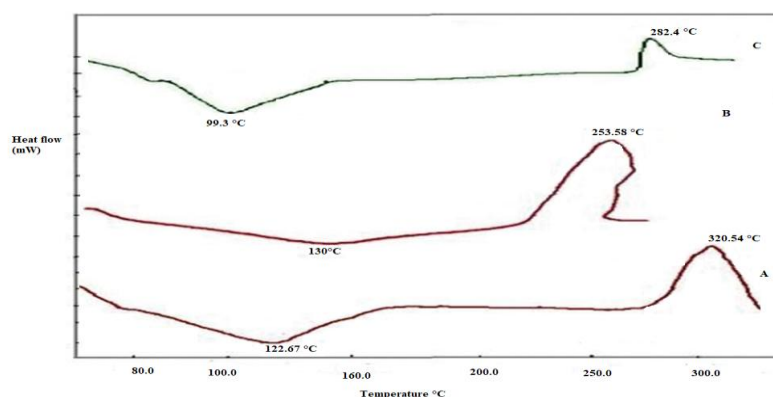


Figure 5. DSC thermograms of various samples to demonstrate the solid state of the drug in the formulation (A: Chitosan; B: Sodium Alginate; C: Optimized Formulation (F5)).

Table 3: Pharmacokinetic parameters of LMWH solution and F1 and F5 (optimized) nanoparticle formulation

Pharmacokinetic Parameter	Clexane (LMWH Marketed product)	F1 Formulation	F5 Formulation
C _{max} (µg/ml)	3.5	15.8	43.8
T _{max} (hrs)	2	4	4
AUC _{0-α} (µg ml ⁻¹ h ⁻¹)	30.2	52.5	148.2

Pharmacokinetic parameters were determined from the plasma concentration-time profile of different formulations (Table 3). For LMWH plain oral solution, the C_{max} value was observed to be 3.5 µg/ml after 2 hrs of oral administration, while administration of Chitosan- LMWH nanoparticles (F1) enhanced the C_{max} 15.8 µg/ml. However, the maximum value was observed for Chitosan-Alginate LMWH loaded PEC nanoparticles, which was 43.8 µg/ml. Similarly, area under the curve (AUC_{0-8 h}) of was increased by 1.7 times for LMWH Chitosan nanoparticles and 4.9 times for Chitosan-Alginate LMWH nanoparticles when compared with a Clexane (LMWH marketed product). This enhanced oral bioavailability was attributed to the interaction of alginate and chitosan which prevented the degradation of drug in -gastric fluid. Chitosan-Alginate LMWH loaded nanoparticles then enter the small intestine where the alginate gets slowly dissolved exposing the amino groups of Chitosan. These amino groups get attached to the negative charge of epithelial lining and help with paracellular transport across the intestinal epithelium. This results in delivery of the drug to systemic circulation without damaging the intestinal epithelium.

The *in vitro* and *in vivo* evaluation of Chitosan-Alginate LMWH nanoparticles demonstrates that the nanoparticulate system can be considered as a useful oral delivery system for enhancing the bioavailability of LMWH.

FTIR spectra of the drug, Chitosan, Sodium Alginate and optimized formulation were recorded in the range of 4000-400 cm^{-1} . The FTIR spectra of drug, Sodium alginate and Chitosan compared with the FTIR spectra of the optimized formulation. The characteristic functional groups of drug and Sodium alginate showed peaks in the following wavenumber. In pure drug there was C-O stretching at 1230 cm^{-1} , N-H stretching at 3350 cm^{-1} , N-H bending at 1624 cm^{-1} . S=O peak at 1145 cm^{-1} . In Sodium alginate characteristic broad peak of O-H was there at 3279-3300 cm^{-1} C-H stretching sharp peak at 2946 cm^{-1} . In chitosan C-H stretching at 3246 cm^{-1} and C-N stretching at 1435 cm^{-1} . 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 optimized formulation (F5) was subjected to DSC studies. DSC confirms the presence of interaction between Chitosan and alginate. As observed from the thermograms shown in Figure.4 the endotherms of CH and SA were exhibited at 111.63°C and 130°C which indicates the evaporation of absorbed water. An endotherm of F5 nanoparticles was observed at

99.6°C which is lower than the endothermic peaks of Chitosan and Sodium alginate individually. This indicates that the hydrophilic groups in the F5 formulation were more exposed possibly due to the formation of gaps after complexation. Exothermic peaks registered in 320.54°C, 253.58°C, and 282.4°C for Chitosan, Sodium alginate and F5 formulation, respectively, indicate that chitosan -alginate LMWH nanoparticles (F5 formulation) have a peak value intermediate between the peaks of Sodium alginate and Chitosan.

CONCLUSION:

In this study, we have fabricated novel oral nanoparticles for low molecular weight heparin (LMWH), optimized the formulation to demonstrate excellent results in relation to the particle size, encapsulation efficiency (81.5%) and *in vitro* drug release for 24 h is about 93 % drug release. Optimized Chitosan-alginate LMWH nanoparticle exhibit 4.9 folds enhancement in AUC when compared to Clexane (LMWH Marketed product) administered orally. These fabricated Chitosan-Alginate LMWH nanoparticles would serve as a promising prospect for the therapy in all the diseases where LMWH is useful.

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