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Biomimetic Solid Lipid Nanoparticles for Oral Bioavailability Enhancement of Low Molecular Weight Heparin and Its Lipid Conjugates: *In Vitro* and *in Vivo* Evaluation

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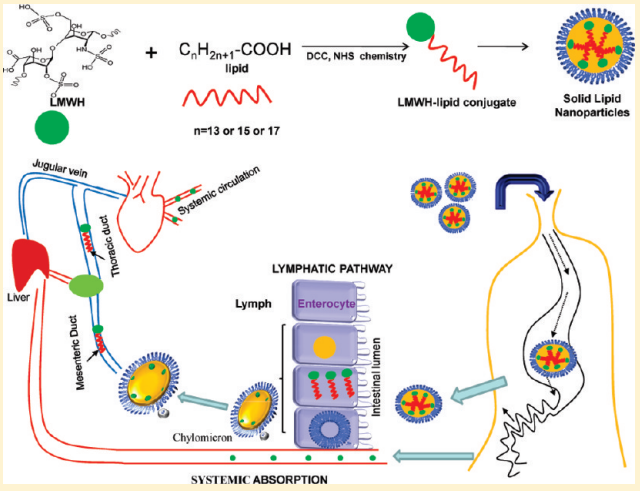
ABSTRACT: Low molecular weight heparin (LMWH) is an anionic oligosaccharide macromolecule, which is commonly administered *via* parenteral routes for the treatment of vascular disorders like deep vein thrombosis (DVT) and pulmonary embolism (PE). Oral heparin delivery can tremendously improve the treatment of such disorders but is restricted due to its large size and anionic character. The present investigation describes synthesis of LMWH−lipid conjugates and their encapsulation in phosphatidylcholine stabilized biomimetic solid lipid nanoparticles (SLNs) for LMWH's oral bioavailability enhancement. Briefly, LMWH was conjugated with different saturated lipids of varying chain length (stearic acid, palmitic acid and myristic acid) using carbodiimide chemistry. The conjugation was confirmed with IR and ^{1}H NMR spectroscopy. The LMWH−lipid conjugate loaded SLNs were characterized for various parameters like shape, size, zeta potential, entrapment efficiency and *in vitro* release behavior in different simulated GIT pH mediums. The GIT toxicity of LMWH−lipid conjugate loaded SLNs to different tissues of intestinal epithelium was observed using H&E staining followed by microscopic observation at cellular level. The LMWH−lipid conjugate loaded SLNs were found to be safe for oral administration. The plasma concentration of LMWH was estimated using anti-FXa chromogenic assay. A significantly higher bioavailability ($p < 0.05$) of LMWH was observed using LMWH−lipid conjugates loaded SLNs in comparison to LMWH loaded SLNs and free LMWH. The order of different conjugates in bioavailability enhancement was LMWH−stearic acid > LMWH−palmitic acid > LMWH−myristic acid. This strategy holds promise for future applications of oral delivery of LMWH conjugates in the form of SLNs particularly for the treatment of cardiovascular disease like DVT and PE.



KEYWORDS: low molecular weight heparin, drug−lipid conjugate, solid lipid nanoparticles, oral delivery, transcellular absorption, anticoagulant

1. INTRODUCTION

Low molecular weight heparin (LMWH) is the agent of choice for treatment of deep vein thrombosis (DVT), pulmonary embolism (PE), coronary syndromes etc.[1] The use of LMWH clinically is limited due to parenteral administration, which necessitates trained personnel for administration, creates pain phobia and is associated with risk of needle borne infections.[2,3] Several attempts have been made to investigate nonparenteral routes (oral, nasal, pulmonary and rectal) of administration for efficient LMWH delivery so as to explore its clinical therapeutics.[4−7] Among all, the oral route is the most preferred and acceptable route of drug delivery from the patient compliance and self-administration point of view.[8] Hence, it demands investigations in the area of oral LMWH delivery.

Oral delivery of LMWH is restricted due to unfavorable gastrointestinal tract (GIT) conditions and physicochemical properties of the drug itself.[9,10] For example, high molecular weight, high anionic charge density and instability of LMWH in the GIT are key problems in its absorption and hence in achieving desired plasma concentration or oral bioavailability.[1] To circumvent these limitations, novel approaches, such as use of penetration enhancers, development of a polymeric carrier and conjugation of hydrophilic LMWH with hydrophobic lipids, have been explored earlier and are in progress in many laboratories, as evidenced by regularly published reports.[7,11−19]

Several penetration enhancers like sodium *N*-[8-(2-hydroxybenzoyl)amino]caprylate (SNAC), *N*-sulfonato-*N*,*O*-carboxymethylchitosan, sodium caprate, glycyrrhetinic acid, zonula

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occludens toxin AT1002 etc. have been reported for delivery of LMWH *via* various noninvasive routes including oral, nasal etc. The penetration enhancers increase absorption of therapeutic molecules via a paracellular route, i.e., open tight junctions.[6,20−25] However, upon oral administration, hepatic metabolism of LMWH cannot be completely avoided by these approaches. This can be possibly overcome by use of lipid carrier systems exploiting lymphatic transport mechanism of dietary lipids.[26]

Solid lipid nanoparticles (SLNs), being lipid based carriers, have been proved for enhanced oral delivery of biomacromolecules such as proteins and peptides to systemic circulation through intestinal lymphatics[26−28] and completely avoid first pass metabolism by the liver. Endogenous stabilizers like phosphatidylcholine derived SLNs resemble, in structural appearance and functions, synthesized lipoproteins like chylomicrometers and hence may be better uptaken by entercoyctes followed by improved oral delivery of loaded content into systemic circulation. However, such systems require a significant lipophilicity in the molecules for their higher loading during preparation and reassembling within the enterocytes and subsequent better absorption into lymphatics. Lipidization of hydrophilic biomacromolecules enhances hydrophobicity and hence makes them suitable for lymphatic transport and improved gastrointestinal absorption.[29] Several lipid conjugates of LMWH have been previously developed and evaluated for its oral bioavailability enhancement.[30]

In the present study, LMWH was conjugated with saturated fatty acid lipids with varying chain length and then subsequently the conjugates were incorporated into SLNs to provide them easy access to intestinal lymphatics and hence their enhanced absorption. The LMWH−lipid conjugate loaded SLNs were characterized for their shape, size, zeta potential, entrapment efficiency and *in vitro* release behavior in various simulated GIT conditions. The toxicological evaluation of developed formulations was conducted on albino rats after oral administration. The plasma concentration profile was estimated using anti-FXa chromogenic assay. The pharmacokinetic parameters were then subsequently calculated on the basis of plasma profile data.

2. MATERIALS AND METHODS

2.1. Materials. LMWH (average molecular weight and anti-FXa activity about 4.35 kDa and 102 IU/mg respectively) was provided as a gift sample by Hebei Changshan Biochemical Pharmaceutical Ltd. (Shijiazhuang, China). Compritol 888 ATO was generously provided by Colorcon Asia Ltd. (Goa, India). Stearic acid, palmitic acid and myristic acid were purchased from Himedia, India. Phosphatidylcholine (PC), dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS) and Sephadex G50 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The anti-FXa acivity of LMWH was estimated by colorimetric assay using a Chromogenix Coatest LMW Heparin Kit (Diapharma, USA). All other chemicals and reagents were of analytical grade unless otherwise specified. Simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.4) were prepared according to the official methods of United State Pharmacopoeia (XXV).

2.2. Synthesis of LMWH−Lipid Conjugates. The amine group of LMWH was conjugated with the carboxylic group of different lipids (stearic acid, palmitic acid and myristic acid) using a previously reported method.[31,32] Briefly, 150 mg of lipid, 165 mg of DCC and 92 mg of NHS were dissolved in 15 mL of DMF. This mixture was kept at room temperature for 5 h under a nitrogen atmosphere; precipitated dicyclohexylurea was filtered off, and free DCC was removed by precipitation followed by dropwise addition of 1 mL of distilled water. Subsequently, the filtered solution was added into 15 mL of distilled water in which excessive NHS was dissolved and activated lipid was precipitated and filtered. LMWH (150 mg) was mixed with activated lipid in a solution of DMF and water (1:1) for 4 h at room temperature. The unreacted activated lipid was then removed by filtration by the addition of water. LMWH−lipid solution was lyophilized, and LMWH−lipid conjugate was obtained as a white powder and preserved until further use.

2.3. Development of LMWH−Lipid Conjugate Loaded SLNs. LMWH−lipid loaded SLNs were prepared by the solvent diffusion method in an aqueous system reported earlier.[26] Briefly, 100 mg of Compritol 888 ATO and 50 mg LMWH−lipid were dissolved in a 10 mL mixture of acetone and ethanol (1:1 v/v, where chloroform was used as cosolvent in minimum amount) maintained at 45 °C temperature in a water bath. This lipid solution was poured into 200 mL of an acidic aqueous phase of 1% w/v PC under continuous mechanical agitation (Remi Instruments, India) with 3000 rpm at room temperature for 10 min. The pH value of the aqueous phase was adjusted to 1.10 by careful addition of 0.1 M hydrochloric acid. The SLN dispersion was quickly produced. The dispersed system was centrifuged at 7500*g* for 30 min using an ultracentrifuge (Hitachi, Japan) and resuspended in distilled water.[33]

2.4. Characterization. *2.4.1. Characterization of LMWH−Lipid Conjugates. 2.4.1.1. Bioactivity Determination of LMWH−Lipid Conjugates.* The LMWH−lipid conjugates were evaluated for their anticoagulant activity anti-FXa assay. Briefly, plasma samples containing standard LMWH were mixed with 200 μL of antithrombin III (AT III) solution (0.1 IU/mL) keeping the AT III concentration in excess in the solution.[4] This solution was further incubated for 2 min at 37 °C followed by addition of 200 μL of FXa (4 μg/mL) solution and again incubated for 1 min. To this resulting solution, FXa substrate (200 μL, 0.8 mmol/mL) was added and again incubated at 37 °C for 5 min. This reaction was stopped by addition of 200 mL of acetic acid (50% v/v). The absorbance was taken at 405 nm for determination of bioactivity and concentration of LMWH in the plasma samples.

2.4.1.2. Partition Coefficient Determination. Partition coefficients of different LMWH−lipid conjugates were estimated in the equal mixture of octanol and water. In brief, 1 mg of LMWH−lipid conjugate was added to 10 mL of a mixture of octanol and water (1:1 v/v ratio). This solution was shaken continuously for 24 h on a wrist shaker (Remi Instruments, India) followed by centrifugation at 4000 rpm for 10 min. The amount of LMWH−lipid conjugate in aqueous medium was determined by anti-FXa chromogenic assay.

2.4.2. Characterization of Carrier System. 2.4.2.1. Morphology. LMWH−lipid conjugate loaded SLNs were examined for their morphology by transmission electron microscope (JEOL, Japan) using a copper grid coated with carbon film. The 1% w/v solution of phosphotungstic acid was used as a negative stain.

2.4.2.2. Size and Zeta Potential. The particle size and zeta potential of SLNs were measured by photon correlation spectroscopy (ZS90 zeta sizer, Malvern Instruments, U.K.) at 25 °C. The samples were kept in polystyrene cuvettes, and observations were made at a 90° fixed angle every time.

2.4.2.3. Entrapment Efficiency. The entrapment efficiency of LMWH in SLNs was estimated by measuring the amount of

Table 1. Bioactivity and Partition Coefficient Values of Different LMWH Lipid Conjugates[a]

formulation	conjugating lipids (carbon chain length)	feed mole ratio	conjugation ratio (no. of lipid molecules/LMWH molecule)	bioactivity (IU/mg)	partition coefficient ($K_{o/w}$)
LMWH				102 ± 0.8	0.0088 ± 0.0012
LMWH−SA	stearic acid (C_{18})	1:15	2.21 ± 0.12	94 ± 3.0	0.0198 ± 0.0028
LMWH−PA	palmitic acid (C_{16})	1:17	2.38 ± 0.14	92 ± 2.2	0.0164 ± 0.0016
LMWH−MA	myristic acid (C_{14})	1:19	2.46 ± 0.15	90 ± 2.6	0.0144 ± 0.0014

[a] Lipid with higher carbon number showed significantly higher ($p < 0.05$) partition coefficient value in comparison to lower carbon number lipids with a slight decrease in anti-FXa activity. Data is presented as mean ± SD ($n = 3$).

unentrapped drug in aqueous solution (supernatant) followed by centrifugation of dispersion using the Azure II colorimetric method reported earlier.[15] In brief, 500 μL aqueous samples were reacted with 4.5 mL of Azure II solution (0.001% w/v) and were estimated spectrophotometrically with a UV spectrophotometer (Cintra 10, Japan) at 530 nm using a standard curve. Entrapment efficiency was calculated using the following equation:

$$\text{entrapment efficiency (EE \%)} = \frac{\text{TD} - \text{FD}}{\text{TD}} \times 100$$

where TD is total drug and FD is free unentrapped drug.

2.4.2.4. In Vitro Release Behavior. The *in vitro* release study was performed in different GIT simulated conditions (SGF pH 1.2 and SIF pH 7.4) using an earlier reported method.[15] Briefly, LMWH−lipid conjugate loaded SLN dispersion was suspended in 10 mL of medium in a flask incubated in a water bath at 37 °C under magnetic stirring at 100 rpm. After regular time intervals, samples were withdrawn followed by replacement with equal volume of fresh medium. The samples were assayed for LMWH using an Azure II based colorimetric method.

2.4.2.5. In Vivo Study. The *in vivo* studies were performed on albino rats of either sex weighing in the range of 100−125 g. The studies were carried out under the guidelines compiled by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animal, Ministry of Culture, Government of India). All the study protocols were approved by the local animal ethics committee. Animals were fasted overnight with free access to water prior to formulation administration.

2.4.2.5.1. Gastrointestinal Toxicological Study. Animals were divided into three groups and received formulations orally with the help of canulla with maximum care and negligible tissue damage. Groups I and II received 0.2 mL of LMWH and LMWH−lipid conjugate loaded SLN formulations respectively. Group III served as control and received an equivalent volume (0.2. mL) of PBS buffer solution pH 7.4. After 1 h of oral administration animals were sacrificed to isolate different GIT tissue sections, which were fixed in neutral buffer formalin followed by microtome processing and slide preparation. A minimum of three animals were screened each time. H&E stained tissue were examined carefully under optical microscope for any tissue damage at 40× (Nikon, Eclipse E200).

2.4.2.6. Plasma Profile and Pharmacokinetic Study. In this study, animals were divided into seven groups. Groups I, II, III and IV received SLNs-LMWH−SA, SLNs-LMWH−PA, SLNs-LMWH−MA and SLNs-LMWH formulations respectively. Groups V and VI received LMWH by oral and intravenous routes respectively. Group VII served as control by receiving PBS solution. Briefly, overnight fasted animals were anesthetized for a very short time with diethyl ether and 0.2 mL of formulation was administered orally with the help of a cannula to each animal in a group. The blood samples were collected from retro-orbital plexus puncture at different time point and were analyzed for LMWH concentration by anti-FXa chromogenic assay as described in section 2.4.1.1. The peak plasma concentration (C_{max}) and the time to reach peak concentration levels (t_{max}) were obtained from the time versus plasma concentration profile. Standard noncompartmental analysis was performed for estimation of absorption profile (Kinetica, Ver 5.0, Innaphase, Corp., Philadelphia, PA, USA). Area under the curve (AUC) was estimated by the linear trapezoidal rule method. The overall bioavailability (F) of LMWH was calculated by comparing its pharmacokinetic data obtained from oral administration with the results obtained from intravenous injection.

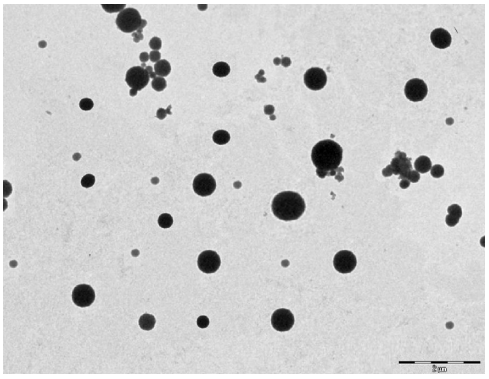

Figure 1. Transmission electron microphotograph of LMWH−lipid conjugate loaded SLNs. Particles were spherical in shape with uniform distribution throughout the dispersion, and the observations were made at least three times for each formulation (bar: 2 μm).

2.4.3. Statistical Analysis. The results were expressed as mean ± standard deviation. Student's t test was conducted on data for statistical analysis, and statistical significance was designated as $p < 0.05$. One-way analysis of variance (ANOVA) followed by post hoc test was applied for multiple comparisons.

3. RESULTS

3.1. Synthesis and Characterization of LMWH−Lipid Conjugates. LMWH was conjugated to stearic acid, palmitic acid and myristic acid following a previously reported method by Lee et al. with slight modification.[32] The carboxylic group of fatty acid was conjugated to the amine group of LMWH. The conjugation was confirmed by amide bond formation using FTIR and ^{1}H NMR. In the FTIR spectra, the peaks at 1720 and 1585 cm^{-1} confirmed the presence of amide bonds in all three LMWH−lipid

Table 2. Characterization of Different LMWH−Lipid Conjugate Loaded SLNs in Terms of Size, Zeta Potential, Polydispersity Index and Entrapment Efficiency[a]

formulation	particle size (nm)	polydispersity index (PDI)	zeta potential (mV)	entrapment efficiency (%)
SLNs-LMWH	280.3 ± 6.8	0.106 ± 0.010	−21.2 ± 1.1	31.3 ± 2.2
SLNs-LMWH−SA	310.2 ± 8.4	0.102 ± 0.004	−24.3 ± 3.6	61.3 ± 5.3
SLNs-LMWH−PA	336.4 ± 5.8	0.114 ± 0.021	−28.5 ± 3.3	62.8 ± 7.2
SLNs-LMWH−MA	376.8 ± 8.6	0.123 ± 0.012	−30.6 ± 4.2	61.8 ± 4.4

[a] Data is presented as mean ± SD (n = 4).

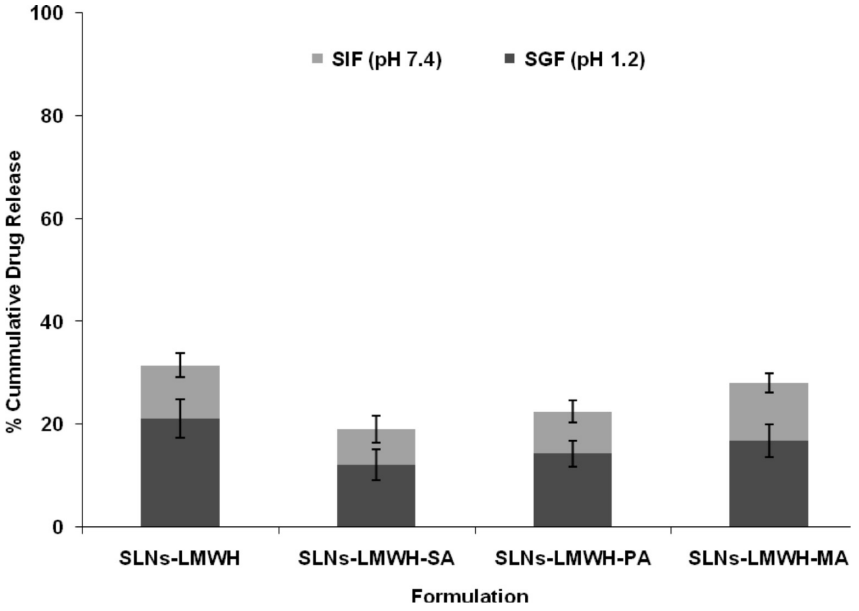

Figure 2. *In vitro* release profile of SLNs in simulated gastric conditions, i.e., SGF (pH 1.2 for 2 h) and SIF (pH 7.4 for 4 h). Dialysis membrane (MWCO 10,000 Da) was used as dispersion bag. Data is presented as mean ± SD (n = 3).

conjugates. This was further supported by NMR study. In the ^{1}H NMR spectra, the amide peak also occurred at 7.58 ppm. This confirmed that LMWH was successfully conjugated with various lipids.

The conjugation ratio, i.e., number of lipid molecules conjugated to one molecule of LMWH, was estimated (Table 1). The conjugation ratios for LMWH−SA, LMWH−PA and LMWH−MA were 2.21 ± 0.12, 2.38 ± 0.14 and 2.46 ± 0.15 respectively. These conjugates of LMWH to lipids were also tested for their retained biological activity in the form of anti-FXa capacity and partition coefficient (Table 1). A decrease in the bioactivity of LMWH−lipid conjugates was observed compared to free LMWH (102 ± 0.0 IU/mg). The LMWH−SA, LMWH−PA and LMWH−MA showed anti-FXa activity about 94 ± 3.0, 92 ± 2.2 and 90 ± 2.6 IU/mg respectively. Partition coefficient values of LMWH−SA, LMWH−PA and LMWH−MA in mixture of octanol:water (1:1 v/v) were found to be 0.0198, 0.0164, and 0.0144 respectively, which were significantly higher (p < 0.05) than that of free LMWH (0.0088).

3.2. Morphology, Size and Entrapment Efficiency of Carrier System. Figure 1 shows a transmission electron microphotograph (TEM) of LMWH−lipid conjugate loaded SLNs. TEM photographs clearly revealed that particles were almost spherical with homogeneous shading with uniformity in particle size. The size and zeta potential of LMWH−lipid conjugate loaded SLNs were determined by photon correlation spectroscopy (Table 2). All formulations possessed mean size range from 280 to 376 nm with PDI value around 0.1 and zeta potential from −21.2 to −30.6 mV. The entrapment efficiency of formulation acts as a determining factor for dose volume that is to be administered for desired therapeutic effect. It was observed that unconjugated LMWH was entrapped nearly about 31.3% (Table 2). However, a significantly high entrapment (p < 0.05), i.e., nearly 2 times higher, was achieved in the case of lipid-conjugated LMWH compared to unconjugated LMWH. At the same time, there was insignificant difference among percent entrapment of different LMWH−lipid conjugates in the SLNs.

3.3. *In Vitro* Release Study. The *in vitro* release behavior of developed formulations was observed in different simulating GIT fluids resembling transit of orally administered formulation (Figure 2). In the case of SGF, a higher release of LMWH from SLNs within 2 h was observed in all cases when compared to release in SIF after 4 h. The release of unconjugated LMWH from SLNs-LMWH was 21.1% and 10.4% in SGF and SIF respectively. However, LMWH when conjugated to lipids showed retarded release in both the mediums. In SGF, formulations SLNs-LMWH−SA, SLNs-LMWH−PA and SLNs-LMWH−MA showed a cumulative release of LMWH, 12.2%, 14.8% and 16.8%, respectively, whereas, in the case of SIF, SLNs-LMWH−SA, SLNs-LMWH−PA and SLNs-LMWH−MA showed a cumulative release of LMWH, 6.8%, 8.2% and 11.2%, respectively.

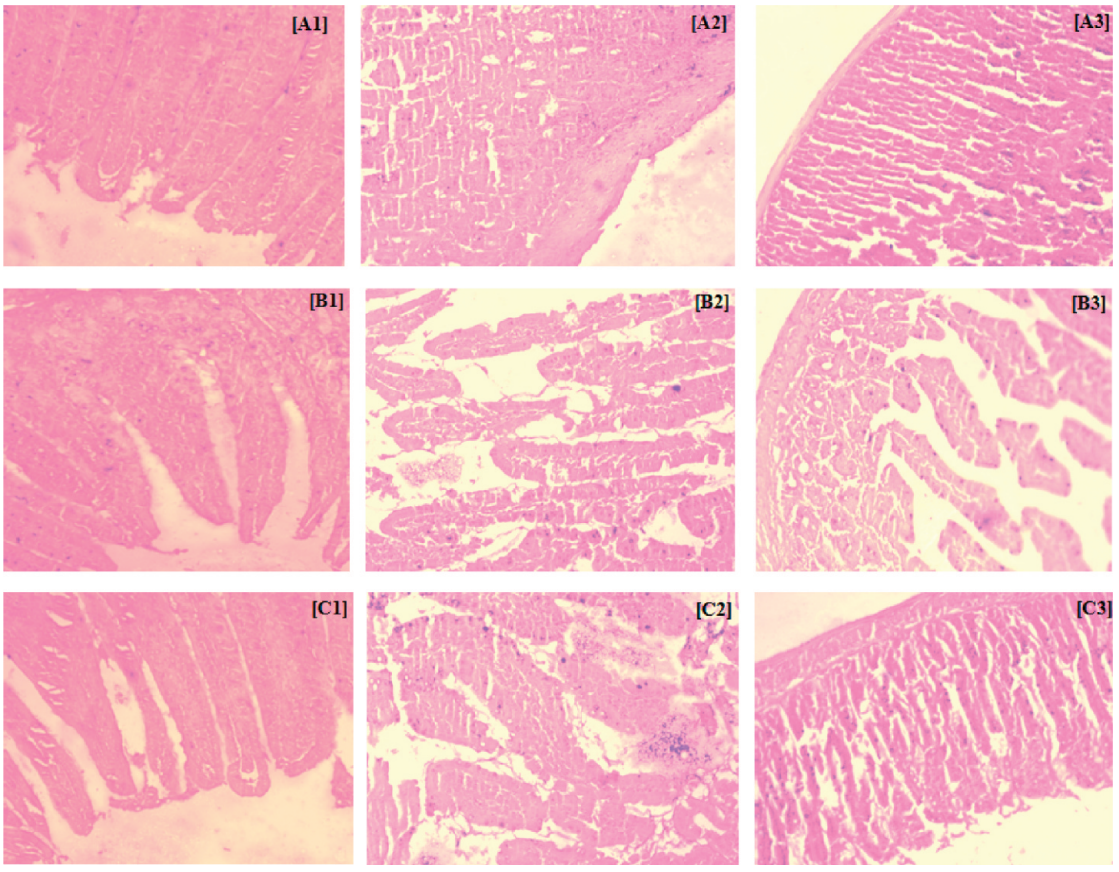

Figure 3. H&E analysis of various GIT tissues after one hour of oral administration of formulations: [A] control; [B] plain LMWH solution in PBS pH 7.4; [C] LMWH−lipid conjugate loaded SLNs. The dose of LMWH was kept constant in both the cases, i.e., 50 mg/kg. The symbols 1, 2, and 3 represent duodenum, jejunum and ileum sections of GIT respectively.

3.4. Histological Examination of Gastrointestinal Tract. Figure 3 shows H&E staining analysis of the various parts of GIT after single dose oral administration of LMWH loaded SLNs in order to identify the extent of disruption caused to these tissues, if any, and also to evaluate safe use of formulation for the purpose. It is clear from Figure 3 that absorption of LMWH along with SLNs did not induce any toxicity to any part of gastrointestinal epithelium, i.e., dudoneum [C1], jejunum [C2] and ileum [C3]. These results were comparable to control. At the same time, no evidence of damage in villi structure was recorded in parts of the duodenum, jejunum and ileum.

3.5. *In Vivo* Study and Pharmacokinetics. The *in vivo* absorption and bioavailability determination was carried out using albino rats after oral administration of different LMWH formulations (Figure 4). The maximum anti-FXa activity of LMWH in plasma was recorded nearly 0.14 IU/mL in 30 min in the case of control LMWH administration. When it was administered in the form of SLNs, the maximum anti-FXa activity recorded was 0.28 IU/mL after 180 min, which was almost double that of free LMWH. The lipid conjugates of LMWH in the form of SLNs showed 0.55, 0.46, 0.42 IU/mL anti-FXa activity in the plasma in the case of SLNs-LMWH−SA, SLNs-LMWH−PA and SLNs-LMWH−MA respectively. The significant anti-FXa activity activity of LMWH was observed even after 360 min of administration in the case of lipid based formulations.

The blood clotting time was also estimated followed by oral administration of different LMWH formulation at different time intervals (Figure 5). The SLNs-LMWH showed significant increase in the clotting time (26 s) in comparison to plain LMWH (14 s). Similarly, LMWH−lipid conjugate based SLN formulations showed a higher clotting time profile than SLNs-LMWH. The SLNs-LMWH−SA, SLNs-LMWH−PA and SLNs-LMWH−MA showed 46, 42, and 39 s clotting time respectively, after 3 h of administration, which remained higher than that of plain LMWH at all time points onward.

Pharmacokinetic parameters were estimated from plasma profile after oral administration (Table 3). SLN-based LMWH formulations significantly enhanced bioavailability of LMWH. This is confirmed from the $AUC_{0\rightarrow 6h}$ value, which was shifted from 27.5 ± 2.1 to 60.9 ± 3.2 μg/mL/min. It was also observed that t_{max} was also changed from 30 to 180 min in the case of all SLN-based formulations. Further, lipid conjugated LMWH loaded SLNs significantly improved the pharmacokinetic parameters. The SLNs-LMWH−SA, SLNs-LMWH−PA and SLNs-LMWH−MA formulations showed $AUC_{0\rightarrow 6h}$ 99.5 ± 3.6, 83.7 ± 2.8 and 73.6 ± 2.2 μg/mL/min, respectively.

4. DISCUSSION

Low molecular weight heparin is a clinically recommended anticoagulant for the treatment of deep vein thrombosis and other cardiovascular disorders.[2] It has minimal side effects and reported drug interactions. However, its use is restricted due to parenteral administration, which is desirable to avoid because of the need of

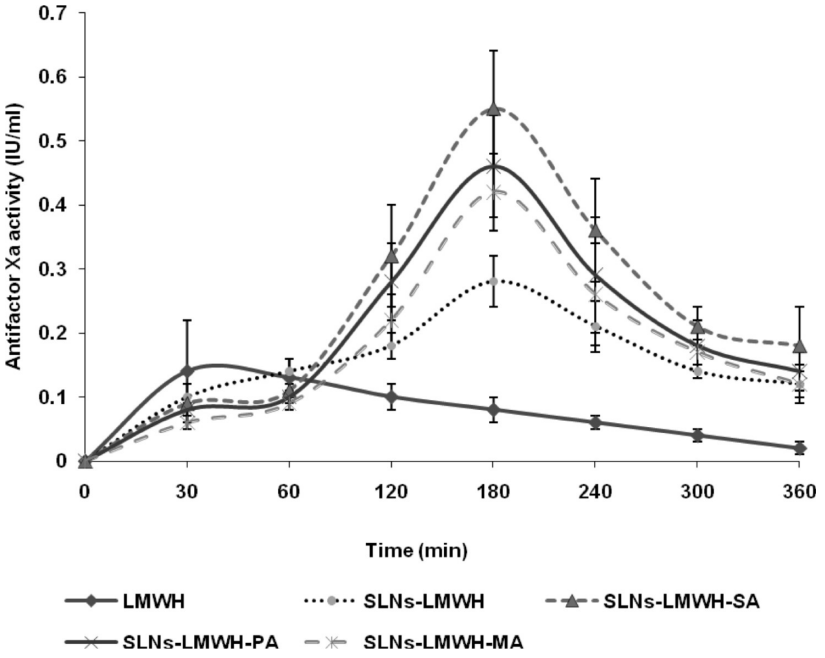

Figure 4. Plasma concentration profiles of different LMWH−lipid conjugate loaded SLN formulations after oral administration. Data is presented as mean ± SD (n = 4).

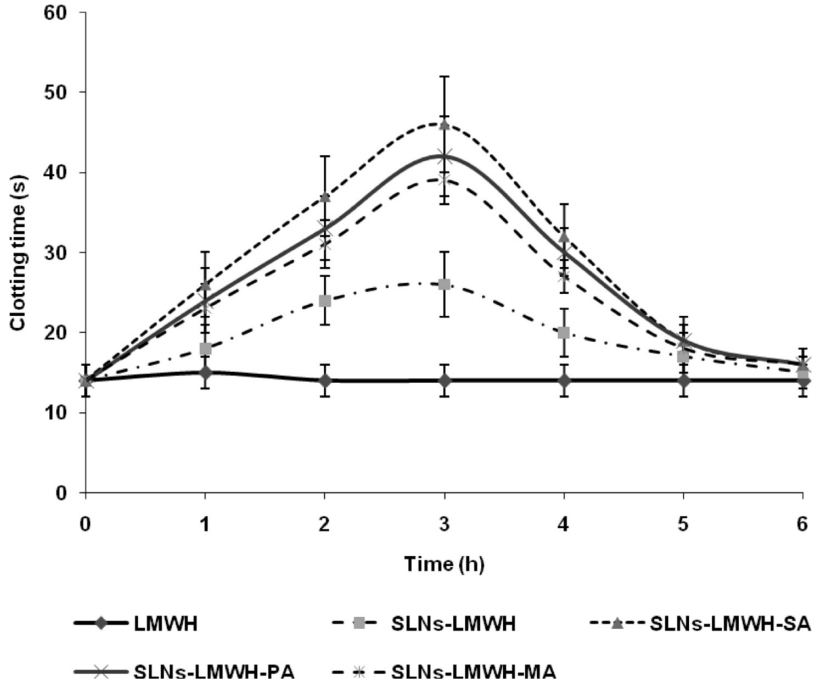

Figure 5. Clotting time profile of different LMWH−lipid conjugate loaded SLN formulations. Data is presented as mean ± SD (n = 4).

skilled personnel and hospitalization, low patient compliance, fear of needle-associated pain and infections etc.[34] Oral heparin formulation may serve as an alternative to both parenteral heparin and presently available oral anticoagulants.[35] Being a large anionic molecule, oral heparin remains less absorbed by intestinal epithelium and hence suffers with problem of low bioavailability.[36] Therefore, strategic delivery of LMWH suitably with the help of an engineered biomimicking novel carrier system (protecting it from degradation and resulting into higher absorption) may be useful for its oral bioavailability enhancement.[37,38] In the present study, three different lipid conjugates of LMWH were synthesized and were further incorporated into PC stabilized SLNs. These chylomicrometer mimicking biocompatible lipid based carrier systems, i.e., SLNs, were expected to follow a transcellular mechanism of lipid transport and hence through intestinal lymphatics may enhance the bioavailability of LMWH.[28]

The amine groups of LMWH were conjugated to carboxylic acid groups of saturated lipids using carbodiimide chemistry. The

Table 3. Pharmacokinetic Parameters of Different LMWH−Lipid Conjugate Loaded SLN Formulations after Oral Administration (Equivalent Dose of LMWH 50 mg/kg)[a]

formulation	dose (mg/kg)	C_{max}(IU/mL)	$t_{max\ (min)}$	$AUC_{0\rightarrow 6h}$ (μg/mL/min)	F (%)	relative bioavailability
LMWH (iv)	1	0.64 ± 0.04		344.7 ± 16.2		
LMWH (oral)	50	0.14 ± 0.02	30	27.5 ± 2.1	0.16	1
SLNs-LMWH (oral)	50	0.28 ± 0.04	180	60.9 ± 3.2	0.35	2.21 ± 0.18
SLNs-LMWH−SA (oral)	50	0.55 ± 0.09	180	99.5 ± 3.6	0.57	3.61 ± 0.23
SLNs-LMWH−PA (oral)	50	0.46 ± 0.08	180	83.7 ± 2.8	0.48	3.04 ± 0.19
SLNs-LMWH−MA (oral)	50	0.42 ± 0.06	180	73.6 ± 2.2	0.42	2.67 ± 0.20

[a] Data is presented as mean ± SD (n = 4).

conjugation was confirmed by characteristics peaks of amide linkages in IR and ^{1}H NMR spectroscopy. These results were similar to previous reports.[30] The lipids were selected on the basis of difference in their carbon chain length. The lipid to LMWH ratio ranged from 2.2 to 2.46 depending upon feed mole ratio used for conjugation. The developed conjugates were evaluated for their retained biological activity and partition coefficient behavior. A significant effect of carbon chain length on bioactivity of the conjugate was observed. Stearic acid (C_{18}) based LMWH conjugates showed better bioactivity and higher partition coefficient value than palmitic acid (C_{16}) and myristic acid (C_{14}) based LMWH conjugates. SLNs were prepared by a solvent diffusion method as it provides reproducibility of formulations with simple laboratory setup.[39] Compritol 888 ATO was used as core material for development of SLNs as it is one of the best materials for lymphatic delivery of bioactives having high entrapment efficiency.[26] TEM images confirmed the presence of spherical nanoparticles with homogeneous shading and distribution in the dispersion. Oral bioavailability of hydrophobic molecules can be enhanced by the intestinal lymphatic route using lipid based systems such as SLNs. Lymphatic transportation of SLNs could be better achieved if they are of nanometric size.[26,27] LMWH and LMWH−lipid loaded SLNs showed nanosize range with acceptable limits of PDI (Table 2). Additionally, zeta potential is an important parameter for study of storage stability particularly in the case of lipid nanoparticles. Sufficiently high zeta potential values of all SLN formulations were recorded, which provided resistance against particle aggregation (Table 2). The entrapment of LMWH in SLNs was measured by an indirect method. A comparatively high entrapment of LMWH−lipid conjugates in the SLNs was observed in comparison to LMWH. This may be attributed to the hydrophobic nature of LMWH−lipid conjugates, which probably favors its entrapment in lipid core matrix of SLNs.

The *in vitro* release study confirmed the suitability of the developed SLNs for oral delivery. In all cases, more than 70% of loaded LMWH was found to be still associated with the formulation even after 6 h transit of formulation from SGF and SIF medium respectively. Negligible toxicological effects of formulations on different parts of GIT (where possible absorption of LMWH loaded SLNs was expected) confirmed the safe use of SLNs for the absorption enhancement of LMWH. A characteristic feature of the developed SLNs was that they were made of nontoxic, biocompatible and biodegradable excipients, i.e., core material (Compritol 888 ATO) and stabilizer (soya lecithin). The anti-FXa activity was measured for determination of plasma concentration of absorbed LMWH after oral administration of the SLN formulations. A significantly higher LMWH remained available in the plasma in the case of SLNs in comparison to plain LMWH solution. However, LMWH−lipid conjugate loaded SLNs showed a comparatively higher bioavailability than LMWH loaded SLNs. This may be attributed to high loading and better lymphatic drainage of LMWH−lipid conjugates and hence enhanced delivery of LMWH into the systemic pool. After 6 h of administration, a sustained release of LMWH in plasma was observed in the case of SLNs, which was otherwise absent in the case of plain LMWH. The absorption of LMWH and its bioactivity in the form of high blood clotting time was also observed using SLNs. The clotting time value was found to be further more in the case of LMWH−lipid conjugate loaded SLNs than LMWH loaded SLNs. The absolute bioavailability (F) of LMWH−lipid conjugate loaded SLNs was nearly 3.6 times higher with increased t_{max} value than that of plain LMWH solution.

5. CONCLUSION

The present study describes the utility of combination approaches, i.e., lipid conjugation and its incorporation into PC stabilized SLNs, in order to develop an oral heparin formulation. Different lipids with saturated hydrocarbons and varying chain length were conjugated to LMWH, which enhanced the hydrophobicity of LMWH in the form of lipid−LMWH conjugates. Incorporation of these conjugates into SLNs significantly improved the bioavailability of LMWH after oral route administration with insignificant toxicity to different GIT tissue. The effect of selected lipid depending upon its chain length and hence hydrophobicity was clearly observed on *in vitro* as well as *in vivo* performance of LMWH. These results open the possibility for testing such biocompatible nontoxic carriers in different clinical conditions like DVT and PE, where oral heparin therapeutics may improve patient compliance.

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