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Development and Validation of RP-HPLC Method for the Determination of Enoxaparin Sodium in Dry Injection Formulation

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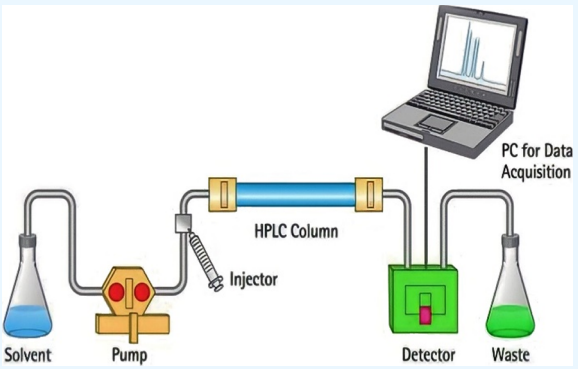
ABSTRACT: Enoxaparin sodium is an anticoagulant medication that is used as a blood thinning agent. It is mostly used for the treatment and prevention of deep vein thrombosis (DVT) and pulmonary embolism (PE). It is also used in certain surgeries and during pregnancy. For the treatment of acute coronary syndrome (ACS) and heart attacks, it may be used. Enoxaparin sodium was validated by the RP-HPLC method. A simple RP-HPLC method was developed in a single HPLC run in a dry powder injection formulation. All injections of HPLC sample were 20 μL volume. The chromatographic separation was completed in the isocratic mode. The used column was USP-L8 (250 mm × 4.6 mm) of BDS type of 10 μm meters in the same mobile phase throughout the analysis by using methanol and ultrapure water with a ratio of 7:93, respectively. The flow rate was 1.0 mL/min. The mobile phase was filtered through 0.45 μm filter paper, and isocratic elution was performed. The refractive index (RI) detector was used to analyze this sample. The specific peak of enoxaparin sodium was observed at 5:56 min. The calculated detection limit (LOD) was 0.351 ppm, and the calculated quantitation limit was 1.063 ppm. In repeatability of precision, the average calculated assay (%) was 100.85%, and the calculated RSD (%) was 0.01. In the accuracy test, the RSD (%) was 0.50, and the mean recovery (%) was 100.35. The system's suitability was within the limit. This newly developed method is proposed according to ICH guidelines, and rules and can be applied effectively for the exact estimation of enoxaparin sodium in injection formulation. This newly developed methodology is affordable in cost as long as less time is taken and the consumption of samples is in smaller quantities for every investigation. In medicinal chemistry, the USP (United States Pharmacopeia) and BP (British Pharmacopeia) are directly involved in production as well as in quality testing.



INTRODUCTION

Enoxaparin sodium (EXP) is chemically called the heparin sodium salt. It is used for the prevention of deep vein thrombosis and pulmonary embolism and the prevention of thrombus formation for the duration of hemodialysis. Sitagliptin (STP) is perceived as (*R*)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3*a*]pyrazin-7(8*H*)-yl]-1-(2,4,5trifluorophenyl)butan-2-amine. It is used for the cure of diabetes. This proposed approach is simple and assesses the above container in a drug measurement's structure.[1] Enoxaparin sodium is a low-weight molecular heparin (LWMH) that is coordinated from heparin sodium of USP grade. It is used as a blood thinner to prevent and cure venous thromboembolism. LWMH is an accurately sulfated direct heteropolymer made out of uric acid (I-iduronic corrosive or D-glucuronic corrosive) gadget exchange and D-glucose amines. Enoxaparin is prepared with the guidance of using substance depolymerization of heparin heteropolymer, which is determined to upgrade its natural leisure action and promote its viability and security profile. The enoxaparin creation was characterized by the composition change in the innermost spine by means of the production of most recent moieties 1,6 hydro bicyclic rings.[2] The normal reaction circumstances and the character of the depolymerizing base contribute to the shape and quantity of unique sequences produced. In addition to the organic pastime profile of the cease product. Significant improvement included the selection of base. For example, the phosphazene base had demonstrated to extrude the pharmacologic pastime product.[3] The prevalent approved by US[4] for the production of enoxaparin via means way of Sandoz and amphastar which were compared with the Sanofi originator enoxaparin in 2015. Two studies, primarily based on totally high-performance liquid chromatography (HPLC)[5] and the second one nuclear magnetic resonance (NMR),[6]

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showed the predominant presence of enoxaparin with unique variations when contrasted with the originator. Enoxaparin sodium is a salt of sodium having low molecular mass heparin.[7] The mechanism of low molecular mass heparin motion is antithrombin dependent. It acts particularly with the aid of using accelerating the charge of positive neutralization-activated coagulation elements with the aid of using antithrombin; however, various mechanisms are involved. The impact of antithrombotic is completely correlated to the inhibition of components Xa-assay which helps to avoid blood clots formation. Ultimately, it helps to avoid the formation of blood clots in deep vein thrombosis, pulmonary embolism, blood vessel headaches, and various kinds of angina or coronary heart attack in the human body. The mechanism of hypercoagulability in patients with COVID-19 is presumably associated with severe endothelial dysfunction and the induction of platelet aggregation[8] In severe COVID-19 patients, changes in several circulating prothrombotic factors improve in factor VIII, an improvement in fibrinogen, and circulation of prothrombotic micro-particles.[9,10] The ensuring blood coagulation issues are taken into consideration to be COVID-19 related coagulopathy.[11] In all sufferers with coagulopathy, thromboprophylaxis with low-weight molecular heparins (LWMH) is suggested to save you from thromboembolic events.[12] HPLC is a separation technique that comprises a small injection volume in a liquid form (in microliters) in a stationary phase which is a compact and fine particle tube. The sample mixture is traveled through the column (packed tube) by the mobile phase (a liquid) under high pressure driven by a pump.[13] The validation in HPLC is to bring out the activity of the sample in the test. Also, for the determination of accuracy in the procedure, efficiency is reproducible of good results. A specificity test is performed to compare the analyte with other potentially present interfering components and compounds. A linearity test is performed to incorporate a satisfactory degree of linearity, accuracy, and precision between lower and upper analyte concentration intervals. Accuracy is performed for defining results to follow standard values. Precision is done under the appropriate conditions; multiple measurements of homogeneous samples are observed. It is carried out in various stages such as ruggedness, repeatability, and reproducibility. HPLC is commonly used in pharmaceutical applications for drug quality research,[14] in environmental applications for biomonitoring for polluting substances,[15] in forensic applications for detection of drug abuse like cocaine in blood,[16] and in food applications for examination of sugar and preservatives in fruit juices and packed milk.[17] The structural formula of enoxaparin sodium is shown in Figure 1.[18]

MATERIALS AND METHODS

Required Materials. Enoxaparin sodium injection (Sandoz), methanol (Sigma-Aldrich), acetonitrile (Sigma-Aldrich), and doubly distilled water (Sandoz pharmaceuticals) were used for the sample preparation and testing. The model of HPLC used Waters 1525, having a gradient pump and a water 2414 refractive index (RI) detector.

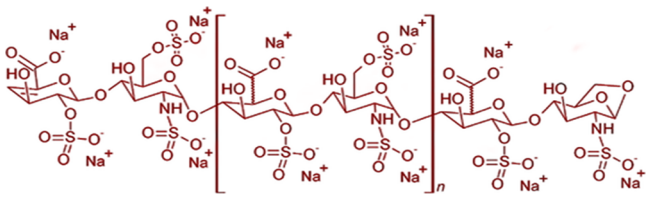

Figure 1. Structural formula of enoxaparin sodium.

Method Development. The solubility of enoxaparin sodium was tested in different solvents. Acetonitrile, methanol, and water were used as solvents for active pharmaceutical ingredient (API) solubility assessment. 1% solution was prepared separately for enoxaparin sodium, and pH was measured, which was calibrated properly for each solution. Double distilled water was used as a diluent. The mobile phase was prepared with methanol and water with a ratio of 7:93, respectively. For the preparation of the standard solution, 100 mg of enoxaparin sodium was added to 20 mL of water in a volumetric flask. It was dissolved and sonicated. The volume was built up by the water process to 20 mL after ensuring the dissolution. The filtration was done by using a 0.45-micro membrane filter paper. For the preparation of the sample solution, 1 mL injection (eq to 100 mg of enoxaparin sodium) was added in 20 mL of water to dilute in a volumetric flask. The solution was filtered through a 0.45-micro membrane buffer. For the chromatographic condition, column [USP-L8 (250 mm × 4.6 mm) 10 μm of BDS type] was used because it gives packing material with an impressive assortment of functional groups the flow rate was 1.0 mL/min to 20 min, and the temperature was 40 °C. The column L8 (250 mm × 4.6 mm) 10 μm of BDS type had a great experience for ideal results among various v/v% compositions of the mobile phase. The desired components were found resolved. The volume of the injection was 20 μL for the development.

Method Validation. According to the guideline of ICH,[19] these are some parameters to be followed for the validation process in HPLC. (1) The specificity test was checked by the blank, sample, and standard solution. These were prepared and injected into the mobile phase of HPLC according to the development portion. (2) In precision, a 100% homogeneous concentration sample solution was prepared in the growth method. Five replicated injections were repeated. In repeatability, six samples were prepared individually at a concentration point of 100%. Three replicates were injected into each sample. The standard deviation was measured and tabulated, and the relative standard deviation percent was calculated. In intermediate precision, composite samples were operated on different days for intermediate accuracy, with another separate analyte holding the same operating conditions. Two separate researchers analyzed six samples' preparations on day one and collected data (%RSD, %Assay). The study was replicated using the alternative analyst with the same chromatographic condition and 100% concentration. (3) For the accuracy test, chemical excipients of which a well-known amount of enoxaparin sodium was evaluated were defined at three different levels of concentrations as followed. For the 80% concentration, 80 mg of enoxaparin sodium was added in 20 mL water. For 100% concentration, 100 mg of enoxaparin sodium was added to 20 mL water. For a 120% concentration, 120 mg of enoxaparin sodium was added to 20 mL water. These samples were evaluated simultaneously in the experimental portion against a prepared sample standard solution. This was measured and recorded as the percentage and mean recovery. (4) For linearity and range, it maintained proportionality (directly) constant in the concentration of analyte in a sample and maintained the range to have accuracy and precision. In stock solution preparation, various concentration level solutions (3000−7000 ppm) are prepared. For

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60% concentration, 60 mg of enoxaparin sodium was added in 20 mL water. For 80% concentration, 80 mg of enoxaparin sodium was added in 20 mL water. For 100% concentration, 100 mg of enoxaparin was added in 20 mL water. For 120% concentration, 120 mg of enoxaparin was added in 20 mL water. For 140% concentration, 140 mg of enoxaparin was added in 20 mL of water. (5) In the detection limit, the maximum level of concentration of a sample was identified but could not be quantified. (6) In the quantitation limit, the maximum concentration of a substance was measured quantitatively accurately and precisely. (7) In robustness, small changes were made in the testing method to check consistency and reliability during routine analysis. The temperature and flow rate were changed for robustness. (8) In system suitability, the six replicates were injected with each solution (standard and sample) with the confirmation of the tests. All parameters were evaluated with the standard parameters.

RESULTS AND DISCUSSION

Specificity. This method is used to analyze and measure precisely the presence of constituents such as excipients, solvents, contaminants, and related chemicals, among others (Table 1). An individual solution of a placebo, mobile phase, API standard, and blank had been injected into the HPLC system and ran for 20 min to assess the selectivity of the proposed analytical approach. No peak was observed in the placebo mobile phase and blank solution shown in Figure 2. Hence, a sharp peak was observed for enoxaparin sodium at 5:54 min because there is no dispersion shown in Figure 3.

Table 1. Specificity Parameters

specificity		
sr. #	name of solution	retention time (min)
1	blank	
2	mobile phase	
3	placebo	
4	enoxaparin sodium	5:54

Linear and Range. Quantitate serial dilution of the stock solution was used to make linearity of five solutions with different concentrations with the order of magnitude given in Table 2, i.e., 3000−7000 μg/mL of enoxaparin sodium solutions in the range of 60−140% (i.e., 60, 100, 120, and 140%) of the working concentration. Each prepared solution was injected with HPLC, and the responses were recorded. The standard solution concentration and its corresponding peak were recorded. Concentrations were plotted on the *X*-axis, and the average peak area was plotted on the *Y*-axis, as shown in Figure 4.

The *Y*-intercept, correlation coefficient, and slope of the regression line (*S*) were calculated and reported. Table lists calibrate the data along with their corresponding standard deviations. The plot for the curve against concentration and peak area was linear in the investigated range (60−140%). The low values of %RSD disclose the method precision. So, the date of linear regression for the calibration curve reveals the ideal relationship against peak area and concentration over an inclusive range.

Detection Limit of Enoxaparin Sodium. The value of the detection limit was estimated based on the slope (*S*) of the calibration curve at points approaching LOD and the response standard deviation of the regression line. The standard deviation of the response was calculated by using the standard deviation of regression line *Y*-intercepts by the formula.[20]

$$\mathrm{LOD} = \frac{3.3 \times \sigma}{S}$$

$$\mathrm{LOD} = \frac{3.3 \times 908.1150147}{8543.166667} = 0.351 \text{ ppm}$$

The calculated limit of detection (LOD) is 0.351 ppm.

Quantitation Limit of Enoxaparin Sodium. The value of the quantitation limit was estimated based on the slope (*S*) of the calibration curve at the point approaching LOQ and the responded standard deviation of the regression line (Table 3). The standard deviation of the response was also calculated by using the standard deviation of regression line *Y*-intercepts here by the following formula:[20]

$$\mathrm{LOQ} = \frac{10 \times \sigma}{S}$$

$$\mathrm{LOQ} = \frac{10 \times 908.1150147}{8543.166667} = 1.063\text{ppm}$$

The calculated limit of detection (LOQ) is 1.063 ppm.

Precision. *Repeatability (Intra-Assay).* Precision or repeatability of the proposed method was conducted by injecting six

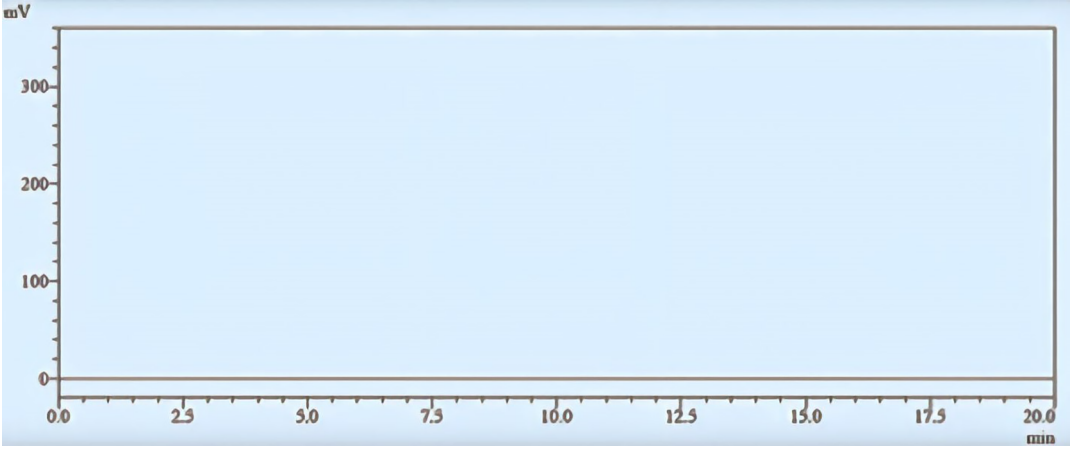

Figure 2. Specific graph of placebo and blank solution.

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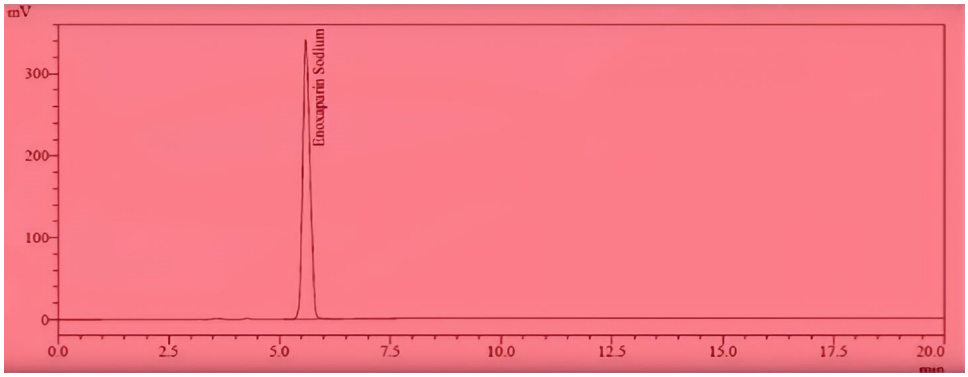

Figure 3. Specific peak of enoxaparin sodium.

Table 2. Linearity and Range Results of Enoxaparin Sodium

linearity and range (enoxaparin sodium)						
sr. #	solution linearity	conc. (ppm)	inj. #	area	average area (n = 2)	RSD (%)
1	60% solution	3000	1	1,230,168	1230375.5	0.02
			2	1,230,583		
2	80% solution	4000	1	1,640,664	1640668.5	0.0004
			2	1,640,673		
3	100% solution	5000	1	2,052,214	2050169.0	0.14
			2	2,048,124		
4	120% solution	6000	1	2,461,864	2461418.5	0.03
			2	2,460,973		
5	140% solution	7000	1	2,870,247	2870360.5	0.01
			2	2,870,474		
slope					8543.1667	
***Y*-intercept**					238.4	
correlation coefficient					1.0000	

Table 3. Summary of the LOD and LOQ Results

validation parameters			
sr. #	API	limit of detection LOD (ppm)	limit of quantitation LOQ (ppm)
1	enoxaparin sodium	0.351	1.063

Table 4. Repeatability Test Results for Enoxaparin Sodium

repeatability (enoxaparin sodium)			
solution	concentration (ppm)	average area	assay (%)
reference	5000	2047746.33	
sample #1		2057243.00	99.9
sample #2		2074495.50	100.7
sample #3		2085055.50	101.2
sample #4		2086920.00	101.3
sample #5		2080004.50	101.0
sample #6		2081856.00	101.1
average assay (%)		100.85%	
% RSD		0.01%	

different samples prepared from a homogeneous blend of marked sample concentrations of 5000 μg/mL standard division (SD), and %RSD values were checked for these tabulated and samples (Table 4).

From the given data, it can be noticed that the relative standard deviation value was found to be <2.0% for the prepared sample. The obtained RSD% value is within the standard acceptable range which confirmed the precision of the suggested method.

Intermediate Precision. The method has been proposed to validate intermediate precision. The composite samples were

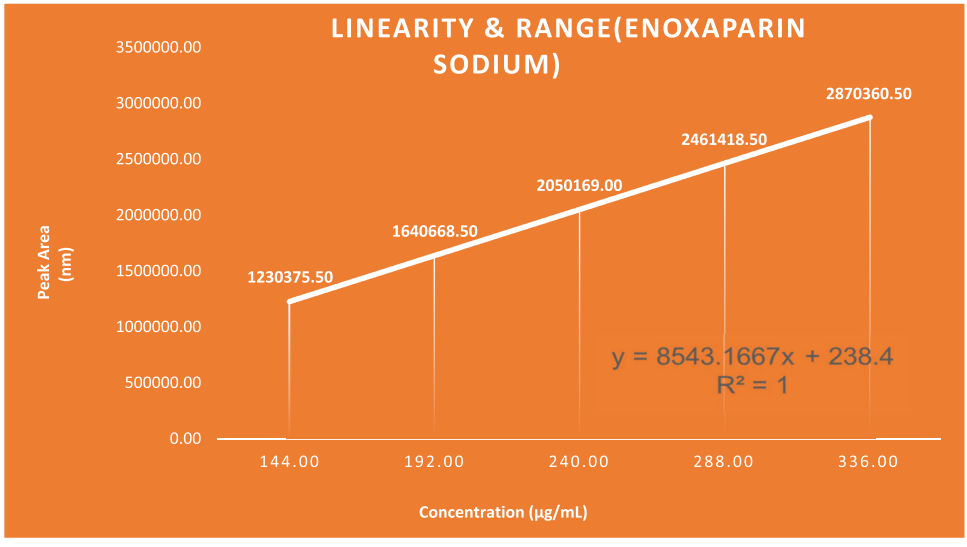

Figure 4. Calibration curve for enoxaparin sodium.

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injected into the HPLC system in intervals of days. Keeping the same operating conditions as described above. Six samples of varying concentrations for each enoxaparin sodium were injected into the HPLC column by two operators on two different days. The difference between the assay value and % RSD for analysis was calculated and tabulated (Tables 5−8).

Table 5. Intermediate Precision (Intra-Day) Results of 1st Analyst on Day-1

intermediate precision (intra-day)			
day-1			
analyst-1:			
solution	concentration (ppm)	average area	assay (%)
reference	5000	2048545.00	
sample # 1		2050547.50	99.50
sample # 2		2053098.50	99.62
sample # 3		2060660.50	99.99
sample # 4		2061798.50	100.04
sample # 5		2061777.50	100.04
sample # 6		2062132.00	100.06
average assay (%)		99.88	
% RSD		0.25	

Table 6. Intermediate Precision (Intra-Day) Results of 2nd Analyst on Day-1

intermediate precision (intra-day)			
day-1			
analyst-2			
solution	concentration (ppm)	average area	assay (%)
reference	5000	2051031.33	
sample # 1		2061471.00	99.91
sample # 2		2062102.00	99.94
sample # 3		2062175.50	99.94
sample # 4		2062535.00	99.96
sample # 5		2062313.50	99.95
sample # 6		2067472.00	100.20
average assay (%)		99.98	
% RSD		0.11	

Table 7. Intermediate Precision (Inter-Day) Results of 1st Analyst on Day-2

intermediate precision (inter-day)			
day-2			
analyst-1			
solution	concentration (ppm)	average area	assay (%)
reference	5000	2048545.00	
sample # 1		2063117.50	100.11
sample # 2		2061695.50	100.04
sample # 3		2064046.00	100.15
sample # 4		2064136.50	100.16
sample # 5		2064003.00	100.15
sample # 6		2064104.00	100.15
average assay (%)		100.13	
% RSD		0.05	

The relative standard deviation %RSD on both days was found to be ≤2% as given in the tables since %RSD for API assay for intermediate precision remains in acceptance criteria.

Table 8. Intermediate Precision (Inter-Day) Results of 2nd Analyst on Day-2

intermediate precision (inter-day)			
day-2			
analyst-2:			
solution	concentration (ppm)	average area	assay (%)
reference	5000	2049452.00	
sample # 1		2065170.00	100.16
sample # 2		2064656.00	100.14
sample # 3		2063977.00	100.10
sample # 4		2063921.00	100.10
sample # 5		2061846.50	100.00
sample # 6		2059088.00	99.87
average assay (%)		100.06	
% RSD		0.11	

Hence, the intermediate precision is an excellent limit for this method.

Accuracy. The accuracy method describes the nearness of the results to be accepted or a standard value. Placebo of the standard of enoxaparin sodium at 80, 100, and 120% concentration. These solutions were injected into the HPLC system under specified chromatographic conditions. The mean of recovered results was carried out for an accurate result (Table 9).

Robustness. In robustness, the small variation in operating conditions to be placed such as flow rate and column temperature. It was changed deliberately to check the consistency and reliability of the test method in routine analysis. Six replicates of each sample of enoxaparin sodium were injected in the HPLC system under chromatographic conditions by varying (i) flow rate ±0.1 mL/min and (ii) column temperature maintained at 35 and 45 °C. The peak responses were recorded to assess the robustness of the developed method in terms of standard deviation and %RSD. The results for each deliberate variation are given in the table (Tables 10 and 11).

Relative standard deviation values at varied parameters for enoxaparin sodium. The obtained results vary within the specified limits, indicating that the analytical method remained unaffected by the deliberately varying values of flow rate and column temperature. Thus, the robustness of the proposed method is established at various experimental conditions.

System Suitability. System suitability tests are conducted to assume that the analyzed instrument, equipment, process, and specimen are suitable for study. Six replicates of the standard solution were injected to perform a system stability check. Retention time, %RSD, theoretical plates, and tailing factor were assessed for the stability of the system and tabulated. All obtained results are within the criteria of acceptance (Table 12).

Acceptance Criteria. The relative standard deviation is ≤2.0%. Theoretical plates should not be less than 2000. The tailing factor should remain at 0.8−2.0.[21]

CONCLUSIONS

For the analysis of enoxaparin sodium by the RP-HPLC method, it is necessary to follow the ICH guidelines. In development, the mobile phase is prepared with methanol and ultrapure water. The standard solution was prepared by adding 100 mg of sodium enoxaparin in 20 mL water. The HPLC

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Table 9. Accuracy Results of Enoxaparin Sodium

accuracy (enoxaparin sodium)							
solution	sample weight (mg)	conc. (ppm)	inj. #	area	average	assay (%)	recovery (%)
reference	100	5000	1	2,048,947	2048428.33		
			2	2,047,654			
			3	2048684			
80%	80	4000	1	1,638,695	1639301.00	80.0	100.03
			2	1,639,907			
100%	100	5000	1	2,049,868	2050000.00	100.08	100.08
			2	2,050,132			
120%	120	6000	1	2,480,848	2480905.00	121.11	100.93
			2	2,480,962			
RSD (%)						0.50	
maximum recovery (%)						100.93	
minimum recovery (%)						100.03	
mean recovery (%)						100.35	

Table 10. Robustness Results of Enoxaparin Sodium (A)

robustness (enoxaparin sodium) A					
variations	inj. #	area	average	assay (%)	RSD (%)
reference	1	2,004,115	2003381.33		0.10
	2	2,001,115			
	3	2,004,914			
flow rate +0.1 mL·min^{-1}	1	2,041,142	2037996.50	101.1	0.22
	2	2,034,851			
	3	2,036,861			
reference	1	2,149,327	2142619.33		0.27
	2	2,139,948			
	3	2,138,583			
flow rate −0.1 mL·min^{-1}	1	2,158,735	2157565.50	100.1	0.08
	2	2,156,396			
	3	2,156,568			
	1	2,110,749			
	2	2,110,659			
	3	2,110,701			

Table 11. Robustness Results of Enoxaparin Sodium (B)

robustness (enoxaparin sodium) B					
variations	inj. #	area	average	assay (%)	RSD (%)
reference	1	2,021,149	2027934.67		0.57
	2	2,021,471			
	3	2,041,184			
tempt 45 °C	1	2,056,184	2055234.50	100.7	0.07
	2	2,054,285			
	3	2,055,235			
reference	1	2,100,418	2104262.33		0.18
	2	2,108,174			
	3	2,104,195			
tempt 35 °C	1	2,110,481	2115815.00	99.9	0.36
	2	2,121,149			
	3	2,115,815			

column USP-L8 (250 mm × 4.6 mm) 10 μm meters were installed. In validation, the sharp peak was reported at 5:54 min in specificity. In linearity and range, the reported slope was 8543.1667, the *Y*-intercept was 238.4, and the correlation coefficient was 1.00. The LOD was 0.351 ppm. The LOQ was 1.063 ppm. In repeatability, the average assay was 100.85%, and the RSD was 0.01%. In intermediate precision, the average assay was 99.88%, and the RSD was 0.25%. In Accuracy, the mean recovery was 100.35%, and the RSD was 0.50%. The findings indicate that the sample has reliable, cost-effective, and quite effective results. All parameters were statistically analyzed using standard deviation and RSD, and the finding was determined to be well within the specified ranges. In the comparison of other analytical methods, the developed HPLC has been found to be more applicable for the determination of the enoxaparin sodium dosage form in a single HPLC run. Results obtained after conducting temperature and photo stress studies indicated the reliability of the proposed method. Therefore, this RP-HPLC method can be applied for enoxaparin sodium quantitative and qualitative analysis of dosage form in pharmaceutical laboratories for routine quality control measurements over a very short interval.

Table 12. System Suitability Results[a]

enoxaparin sodium				
sr. #	retention time (min)	area	theoretical plates	tailing factor
1	5.5	2,013,850	23,098	1.100
2	5.53	2,019,483	23,425	1.100
3	5.56	2,048,124	23,557	1.110
4	5.54	2,015,747	23,146	1.100
5	5.55	2,005,935	23,352	1.110
6	5.52	2,005,726	23,333	1.110
average	5.54	2018144.17	23318.500	1.105
SD	0.005	15671.910	172.01	0.005
% RSD	0.06	0.78	0.74	0.50

[a]System suitability results are within limits.

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Notes

The authors declare no competing financial interest.

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