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DETERMINATION OF ASSAY AND CHROMATOGRAPHIC PURITY OF FONDAPARINUX SODIUM BY SAX HPLC

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ABSTRACT

A new, simple, sensitive and accurate SAX HPLC method was developed for determination of Fondaparinux sodium in Fondaparinux sodium injection. This method can be used for determination of assay and chromatographic purity of Fondaparinux sodium in Arixtra and other injectable formulations containing Fondaparinux sodium. Chromatographic separation was achieved using a high quality polystyrene-divinyl benzene resin based SAX column (with covalent bonded trimethylammonium anion exchange groups); a linear gradient elution from 10% to 41% of solution-B, which is 0.5 M lithium perchlorate containing 5 mM disodium hydrogen phosphate at pH 6.8 and solution-A being 5 mM disodium hydrogen phosphate at pH 2.5 and a UV detection at 210 nm was employed. The method has been validated and was found to be precise and accurate for determination of both assay and chromatographic purity of Fondaparinux sodium in Arixtra injection. The limit of detection for Fondaparinux was found to be 3.3 µg/mL, which is 0.06% with respect to the method concentration of 5.0 mg/mL. The method was found to be linear over the range of 50-150% of the method concentration. The precision, accuracy, specificity, robustness and solution stability were determined.

Key words: Arixtra injection, Fondaparinux sodium, Lithium perchlorate insitu, pH-gradient, SAX HPLC, Synthetic heparin.

INTRODUCTION

Heparin sodium is the sodium salt of sulfated glycosaminoglycans containing a heterogeneous mixture of repeating uronic acid and N-sulphated glucosamine groups of varying molecular weights[1,2] (Fig. 1). The commercially available Heparin sodium is isolated from the porcine or bovine intestinal mucosa and hence is more susceptible to contamination and impurities resulting from the animal origin[3]. Heparin is an antithrombotic drug used in surgery. The antithrombin binding site of heparin is the sulfated

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pentasaccharide moeity[4-6]. Scientists have isolated this moiety and developed a synthetic process, which comprises of over 50 steps and named it as Fondaparinux sodium (Fig. 2), which is also called as synthetic Heparin[7,8] as it is devoid of any impurities, which usually result from animal source[9].

We have been working on the analytical method development for determination of impurities in Heparin sodium using SAX HPLC[10-14]. We extended our analytical research towards synthetic heparin and we could find only one published literature[15] for the determination of impurities in Fondaparinux sodium, which is also now available in the USP PF[16]. Analytical method development was initiated to develop a method for determination of both assay and impurities in Synthetic Heparin viz., Fondaparinux sodium. Commercial samples of Fondaparinux sodium available in the market viz. Arixtra injection was used for carrying out the development experiments.

The tests for assay and purity present in the USP PF (pharmacopeial forum) uses DMSO and a high salt concentration of up to 2.0 M sodium chloride in the mobile phase, a polyvinylbenzyl ammonium (divinyl benzene) resin based SAX column with medium-low hydrophobicity. The use of DMSO affects the column life, high salt concentration leading to high back pressures in the HPLC system and a high drift and poor resolution of impurities was observed with the presently used SAX column.

Hence, we conducted further method development experiments to produce a more simple and sensitive method, which would yield a better resolution of the active and the impurities. The experiments resulted in a pH gradient SAX-HPLC method, which uses lithium perchlorate, (which is prepared in situ by treating lithium hydroxide with perchloric acid) mobile phase, which decreased the drift in the baseline significantly facilitating better limit of detection and also improved the resolution between Fondaparinux sodium and the closely eluting impurities. The limit of detection of Fondaparinux in this method was 0.06% (i.e. 3.3 µg/mL) with respect to the method concentration of 5.0 mg/mL. The HPLC method developed employs a strong anion exchange column containing a high quality polystyrene-divinyl benzene resin with covalent bonded trimethylammonium anion exchange groups and a gradient elution of 0.5 M lithium perchlorate (*in situ*) mobile phase containing 5 mM phosphate buffer and adjusted to a pH of 6.8 (solution-B) and 5 mM phosphate buffer at pH 2.5 (solution-A). A UV detector set at 210 nm was used and the sample solutions are prepared in 0.9% w/v sodium chloride solution in water, the diluent. The sample concentration is 5.0 mg/mL and an injection volume of 100 µL is used. The analytical method was validated and was found to be precise, accurate and linear over a range of 50-150% of the method concentration.

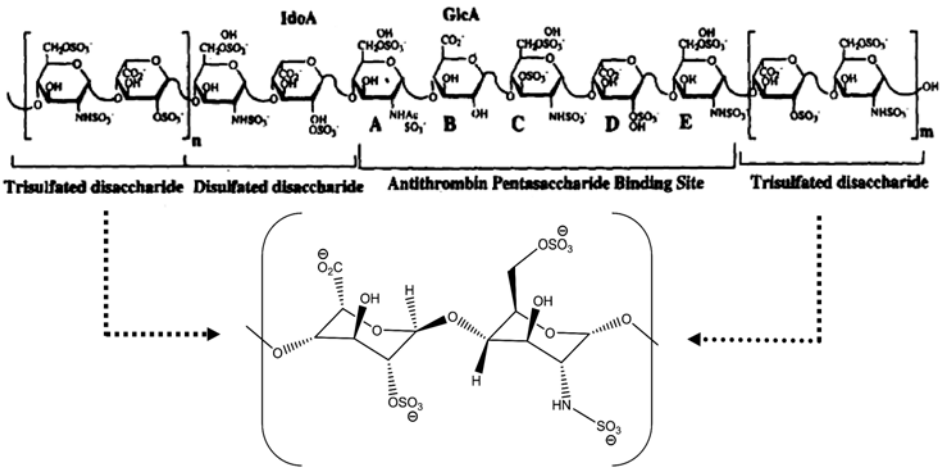

Fig. 1: Chemical structure of heparin sodium

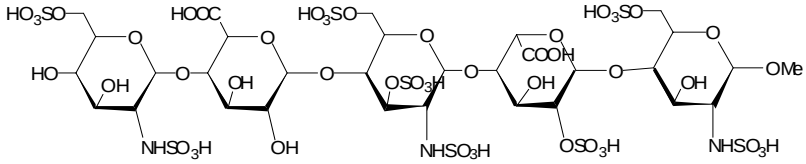

Fig. 2: Chemical structure of fondaparinux sodium (Synthetic heparin)

EXPERIMENTAL

Materials and methods

Reagents and materials

Commercial samples of Fondaparinux sodium injection, viz. Arixtra, B. No: 7171; 7165; 7180; 7196 were procured from the market. Fondaparinux sodium standard sample was obtained from Gland Pharma Limited, Dundigal, Hyderabad. Sodium hydroxide pellets, extrapure, Lobachemie; Sodium dihydrogen phosphate monohydrate GR, Merck; Lithium hydroxide anhydrous, Laboratory grade, Fisher Scientific; Perchloric acid about 70%, ACS, Merck; and Orthophosphoric acid, HPLC grade, Fisher Scientific were used.

Instrumentation

A Shimadzu LC 2010 A model HPLC equipped with gradient pump, eluent degasser, autosampler, column oven and UV detector was used. Data acquisition was performed with a Shimadzu LC solutions software/chromatography data processor (Shimadzu Corporation, Japan).

Chromatographic conditions

A Hamilton SAX column (250 mm length x 4.6 mm id) was used for the determination of assay and chromatographic purity of Fondaparinux sodium in Arixtra injection. The mobile phase was a mixture of solution A – 5 mM disodium hydrogen phosphate (pH adjusted to 2.5 with phosphoric acid) and solution B – 0.5 M lithium perchlorate (prepared in situ) containing 5 mM disodium hydrogen phosphate (pH adjusted to 6.8 with lithium hydroxide). A linear gradient from 10 to 41% of solution B was used over a period of 50 min, return to 10% solution B at the end of 51 min and a stabilization period of 9 min at 10% solution B, thereby resulting in a 60 mins run time. The flow rate was 1.0 mL/min. The column temperature was maintained at 30°C. The sample concentration was 5.0 mg/mL in diluent (0.9% w/v sodium chloride solution in water), injection volume was 100 µL and the wavelength of detection was 210 nm.

Solution preparation

Mobile phase preparation

Solution A**:** Accurately weigh about 710 mg of disodium hydrogen phosphate, dissolve and make up to 1000 mL with MQ water and adjust the pH to 2.5 with phosphoric acid. Filter the solution through a 0.45 µ membrane and sonicate to degas. Solution B: Accurately add about 43 mL of 70% perchloric acid to about 250 mL of MQ water, perform the addition gradually and mix slowly while addition. Accurately weigh about 12 g of anhydrous lithium hydroxide, add about 500 mL of MQ water and dissolve. Add the perchloric acid solution slowly and dropwise to the lithium hydroxide solution and mix well. To this solution add about 710 mg of accurately weighed disodium hydrogen phosphate and sonicate to dissolve. Make up the volume to 1000 mL with MQ water and adjust the pH to 6.8 with dilute lithium hydroxide solution. Filter the solution through a 0.45 µ membrane and sonicate to degas.

Diluent

0.9% w/v sodium chloride in water.

System suitability solution preparation

To 2 mL of standard preparation for assay, add 0.05 mL of 0.01 N HCl and autoclave the solution for 30 min at 121^{o}C.

Sensitivity check solution (0.01 mg/mL)

Dilute 0.2 mL of standard preparation for assay to 10.0 mL with diluent. Accurately

pipette out 1.0 mL of this solution into a 10 mL volumetric flask and dilute to volume with diluent.

Standard preparation for assay

Accurately weigh about 50 mg of Fondaparinux standard in a 10 mL volumetric flask and dilute to volume with diluent.

Sample preparation

Dilute 2.0 mL of Fondaparinux sodium injection (Arixtra-12.5 mg/mL) to 5 mL with diluent to obtain a sample concentration of 5 mg/mL.

RESULTS AND DISCUSSION

Development of chromatographic method

Optimization of mobile phase

Perchlorate and its concentration

The use of perchlorate salt in the mobile phase is known to enhance the UV absorption of compounds, which do not have significant wavelength of absorption. We know from our earlier studies that the *in situ* preparation of perchlorate mobile phase is capable of reducing the drift in the baseline significantly (Fig. 3). Different perchlorates at different concentration levels were evaluated (Fig. 4 & Fig. 5) and it was found that 0.5 M lithium perchlorate yielded better separation than others (Fig. 6).

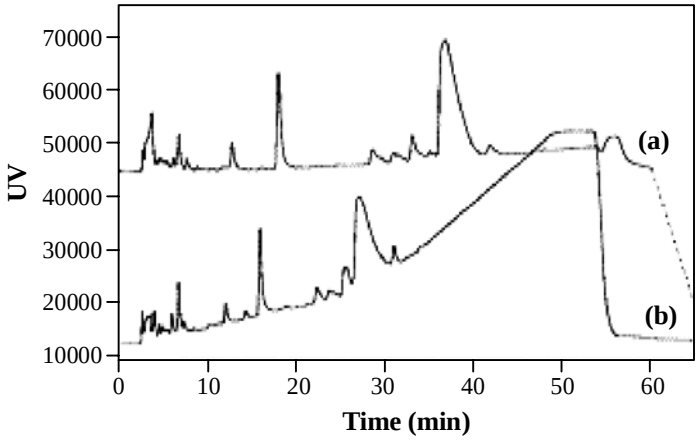

Fig. 3: Comparison of baseline drifts in System suitability chromatograms obtained with (a) $LiClO_4$ - *In situ* and (b) $NaClO_4$ - Commercial mobile phases

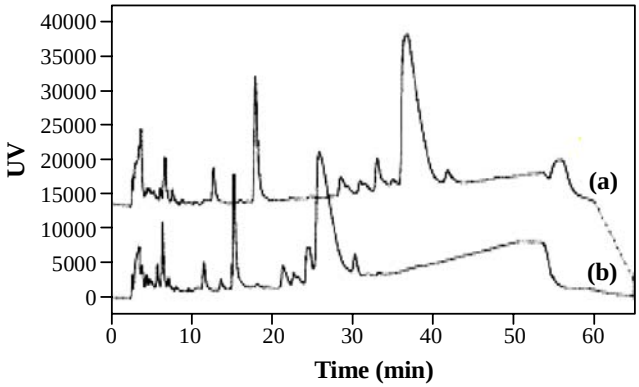

Fig. 4: Comparison of system suitability chromatograms obtained with (a) $LiClO_4$ (*in situ*) and (b) $NaClO_4$ (*in situ*) mobile phases

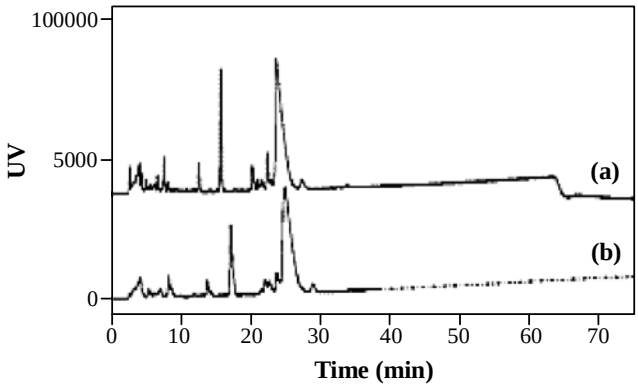

Fig. 5: Comparison of system suitability chromatograms obtained with (a) $LiClO_4$ (*in situ*) and (b) NH_4ClO_4 (*in situ*) mobile phases

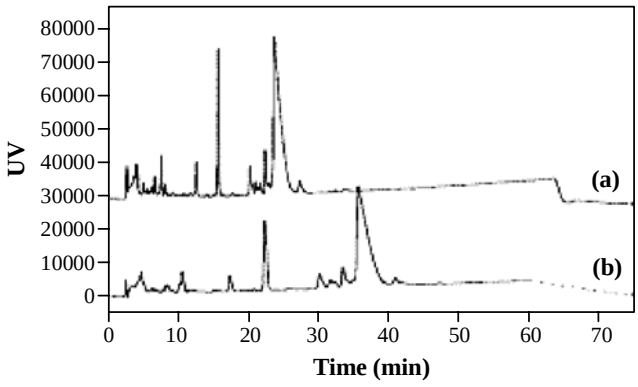

Fig. 6: Comparison of system suitability chromatograms obtained with (a) 0.8 M $LiClO_4$ (*in situ*) and (b) 0.5 M $LiClO_4$ (*in situ*) mobile phases

Phosphate buffer and its concentration

After several trials, 5 mM disodiumhydrogen phosphate was finalized for the phosphate buffer in solution-A and solution-B. The disodium salt was preferred instead of sodiumdihydrogen phosphate as the former yielded sharper peaks and better resolution probably due to its greater ionic strength (Fig. 7, 8 & 9).

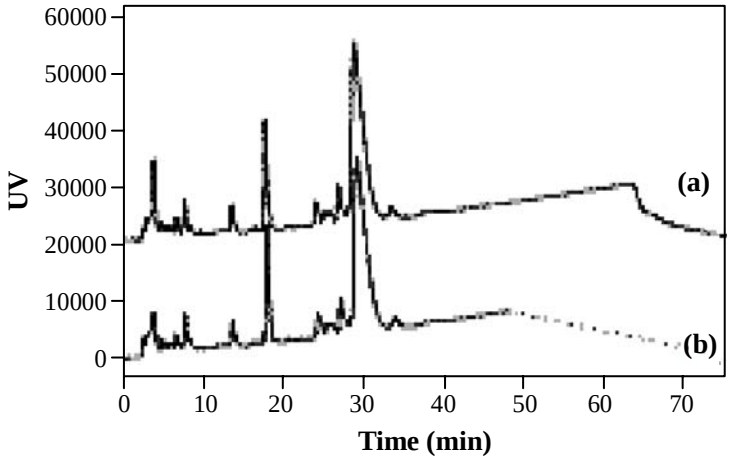

Fig. 7: Comparison of system suitability chromatograms obtained with (a) Na_2HPO_4 and (b) NaH_2PO_4

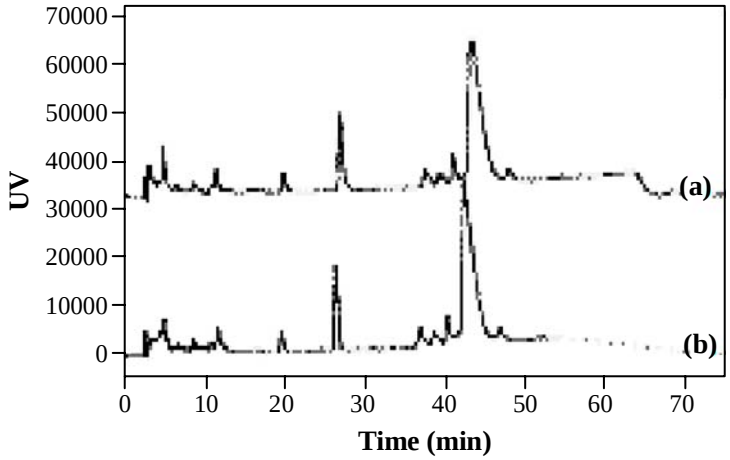

Fig. 8: Comparison of system suitability chromatograms obtained with (a) 5 mM Na_2HPO_4 and (b) 2.5 mM Na_2HPO_4

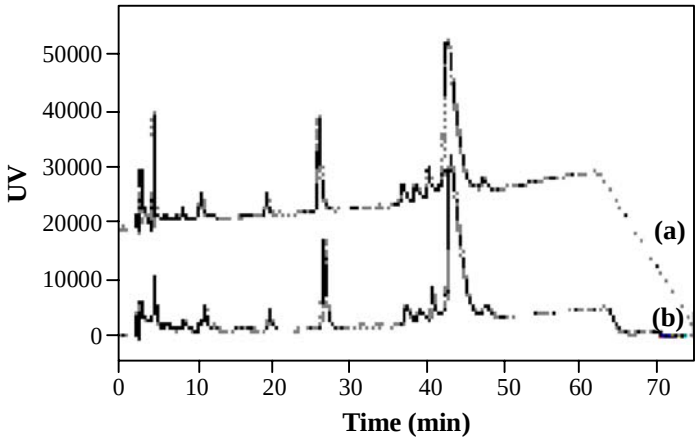

Fig. 9: Comparison of system suitability chromatograms obtained with (a) 10 mM Na_2HPO_4 and (b) 5 mM Na_2HPO_4

pH of solution-A and solution-B

Originally an acidic pH of 2.5 was chosen for both solution-A and B. However, experiments with higher pH values established that the separation was better when a pH gradient was opted. Hence, a pH of 2.5 for solution-A and a pH of 6.8 for solution-B was finalized (Fig. 10).

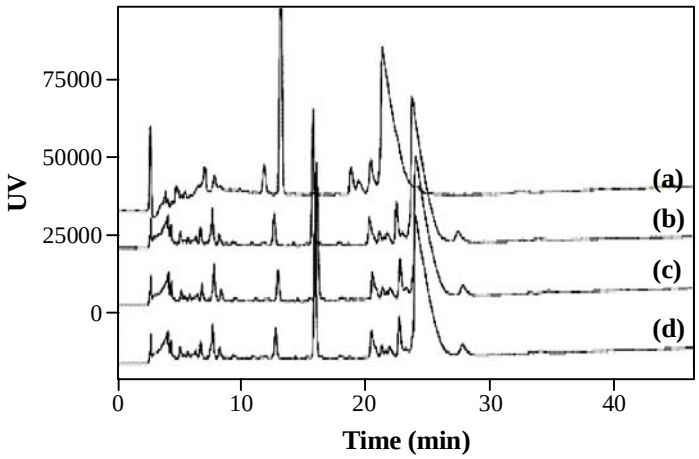

Fig. 10: Comparison of system suitability chromatograms obtained with pH variations in mobile phase

(a) Sol. A and B pH 6.8; (b) Soln A pH 2.5; sol. B pH 6.8; (c) Sol. A pH 2.5; sol. B pH 5.0; (d) Sol. A and B pH 2.5

Gradient optimization

Several trials were performed to optimize the gradient program as it was very critical to achieve a good resolution between the main peak and the closely eluting impurities at 0.9 and 1.1 RRTs (Fig. 11). The initial percentage of 10% solution-B in the gradient program is very critical, and a linear gradient from 10 to 41% of solution B was used over a period of 50 minutes, return to 10% solution B at the end of 51 minutes and a stabilization period of 9 minutes at 10% solution B, thereby resulting in a 60 minutes run time. The repeatability was checked with the finalized gradient program by replicate injections of system suitability solution (Fig. 12).

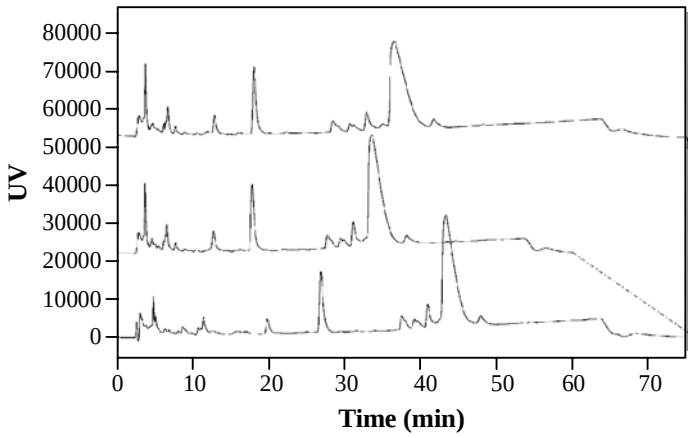

Fig. 11: Comparison of system suitability chromatograms obtained with variations in gradient program

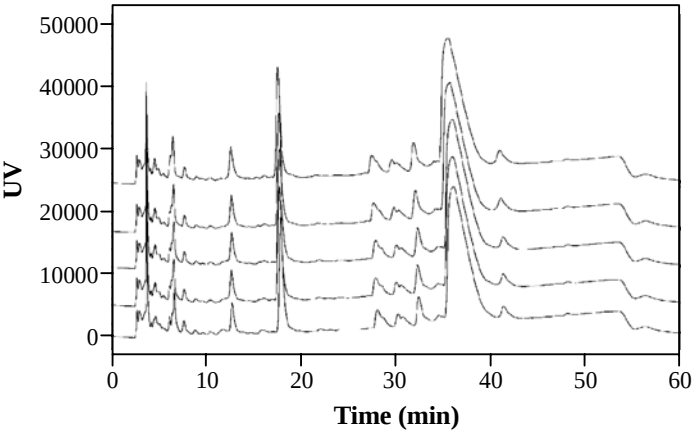

Fig. 12: Overlay of system suitability chromatograms obtained in the finalized gradient program to establish repeatability

Wavelength of detection

As Fondaparinux was found to have maximum response at 210 nm when compared to other wavelengths, 210 nm was chosen as the wavelength of detection for this method (Fig.13).

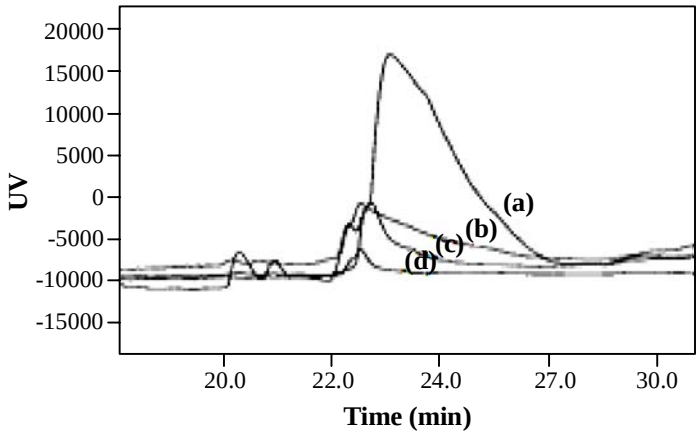

Fig. 13: Comparison of UV responses obtained for Fondaparinux peak at (a) 210 nm (b) 225 nm (c) 232 nm (d) 254 nm

Column temperature

A column temperature of 30^{o}C was chosen after evaluation of the resolution criteria and the peak shapes at other column temperatures (Fig. 14).

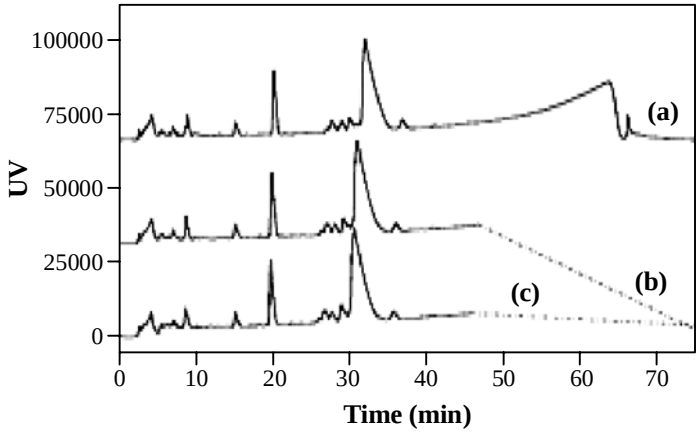

Fig. 14: Comparison of system suitability chromatograms obtained with variations in column temperature (a) 40^{o}C (b) 35^{o}C (c) 30^{o}C

Method validation

The optimized SAX-HPLC method was validated according to ICH guidelines[17]. The validation experiments demonstrate, range (accuracy and linearity), precision, linearity, specificity, limit of detection and quantification, precision at limit of quantification, precision and accuracy at limit of quantification, solution stability and robustness. System suitability parameters were also evaluated.

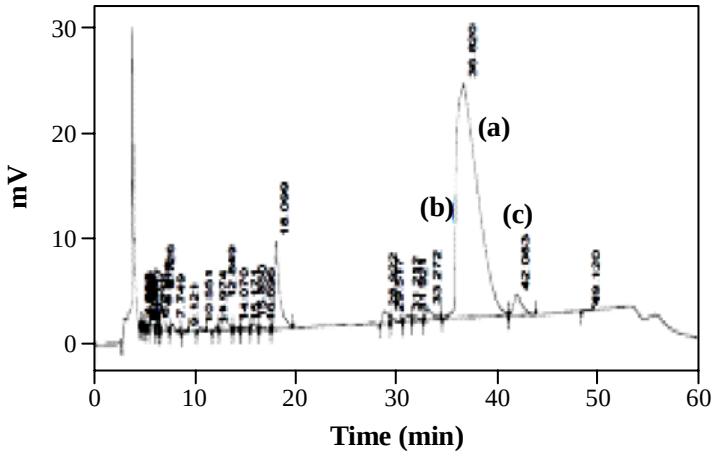

Fig. 15: Typical system suitability chromatogram with peaks corresponding to (a) Fondaparinux (b) 0.9 RRT Impurity (c) 1.1 RRT Impurity

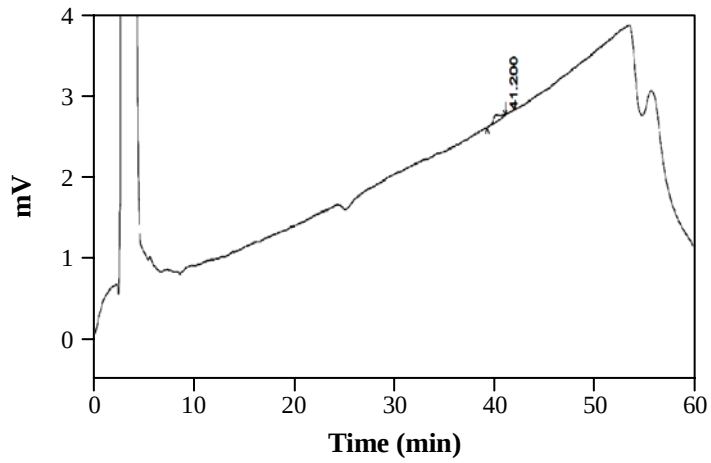

Fig. 16: Typical sensitivity solution chromatogram containing 0.01 mg/mL of fondaparinux sodium

System suitability test

The acceptance criteria set was: A detectable peak of Fondaparinux should be obtained with the sensitivity solution with a signal to noise ratio of not less than 10; the resolution between the impurity at 0.9 RRT and Fondaparinux should be not less than 1.0 in the chromatogram obtained with system suitability solution; and the resolution between the impurity at 1.1 RRT and Fondaparinux should be not less than 1.5 in the chromatogram obtained with system suitability solution. The results obtained were within acceptable limits. Typical system suitability chromatogram and the sensitivity solution chromatogram are shown in Fig. 15 & Fig. 16.

Range (Accuracy, Precision & Linearity)

Accuracy: Recovery solutions were prepared at concentrations spanning from 50 to 150% of the test concentration of 5.0 mg/mL. The % recovery for Fondaparinux was calculated for the individual runs at each level and a mean of the recovery was determined based on the un-rounded data and reported to one decimal place. The accuracy (recovery) results are presented in Table 1. The overlay of range chromatograms is presented in Fig. 17. Accuracy at each level was within the acceptance criteria of 80.0-120.0% of the theoretical concentration.

Table 1: Results of accuracy study

Level	Accuracy (% Recovery)
50%	110.14
75%	110.12
100%	110.88
125%	111.09
150%	111.61

Precision: Repeatability was determined by analyzing the sample preparation containing Fondaparinux sodium at 100% level of the method concentration. The assay content of Fondaparinux and the chromatographic purity was determined for each of the run and the mean of the results were determined individually for assay and chromatographic purity. As shown in Table 2, percent RSD for the assay and chromatographic purity results of six runs (containing Fondaparinux at 100% method concentration) was not more than 15.0%. Therefore, the method is precise and repeatable.

Table 2: Precision results

S. No.	Level	Run	Assay (%)	Chromatographic purity			
				Main peak purity	Single max. imp. (0.49 RRT)	Impurity at 1.1 RRT	Total impurities
1	100%	1	110.52	97.12	1.62	0.35	2.88
2	100%	2	110.53	96.99	1.64	0.35	3.01
3	100%	3	110.92	97.00	1.64	0.35	3.00
4	100%	4	110.63	97.08	1.66	0.36	2.92
5	100%	5	111.71	97.09	1.61	0.35	2.91
6	100%	6	110.95	96.96	1.64	0.36	3.04
	Average		110.88	97.04	1.63	0.35	2.96
	% RSD		**0.41**	**0.06**	**1.05**	**2.01**	**1.98**

Linearity: Linearity was performed as a part of range study to assess whether a linear relationship is obtained between the response and the concentration for the analyte over the intended operating range of the method. A minimum of five concentration levels were analyzed over a range of 50 to 150% of the method concentration. A linear regression analysis (without forcing through the origin) was performed on the data (concentration and peak response). The individual data points (Table 3) and plots of response versus concentration (Fig. 18) are presented below. Index of determination (r^2) was found to be 0.9995.

Table 3: Linearity results

Level (with respect to method concentration)	Concentration in (mg/mL)	Response (Area)
50 %	2.50	1564082
75 %	3.75	2345798
100%	5.00	3149209
125%	6.25	3944051
150%	7.50	4754856

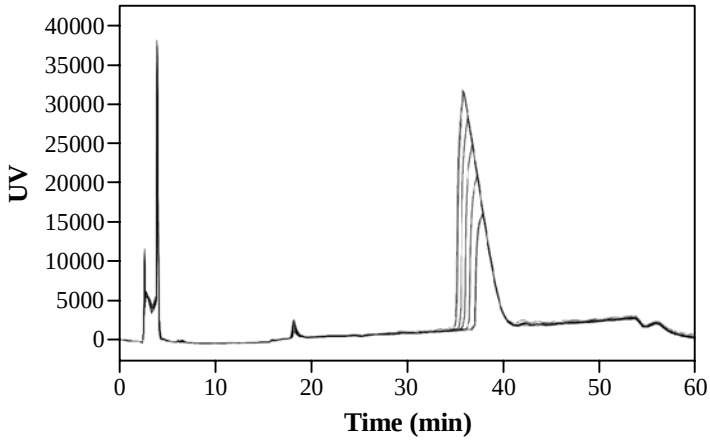

Fig. 17: Overlay of chromatograms of Fondaparinux (Arixtra) over the range establishing precision, accuracy and linearity

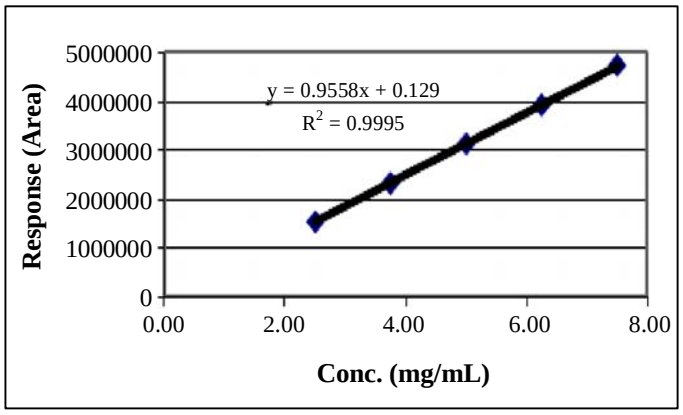

Fig. 18: Linearity plot

Specificity

Specificity was evaluated to ensure that no other compounds that may be present interfere appreciably with the quantitation of the analyte. Specificity of the method was demonstrated by its ability to separate Fondaparinux sodium from its impurities. It was found that there were no peaks due to the diluent-blank or the impurities interfering with the quantification of Fondaparinux sodium (Fig. 19).

Limit of detection and Limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) was established by analyzing the solutions of Fondaparinux prepared at different concentrations, ranging from

2.0% to 0.05% with respect to the test concentration of 5.0 mg/mL. The overlay of LOD chromatograms is presented in Fig. 20. The limit of detection and limit of quantification of Fondaparinux were determined using the S/N ratio method. The LOD and LOQ obtained for Fondaparinux were 0.066% and 0.20% (with respect to the test concentration of 5.0 mg/mL), respectively.

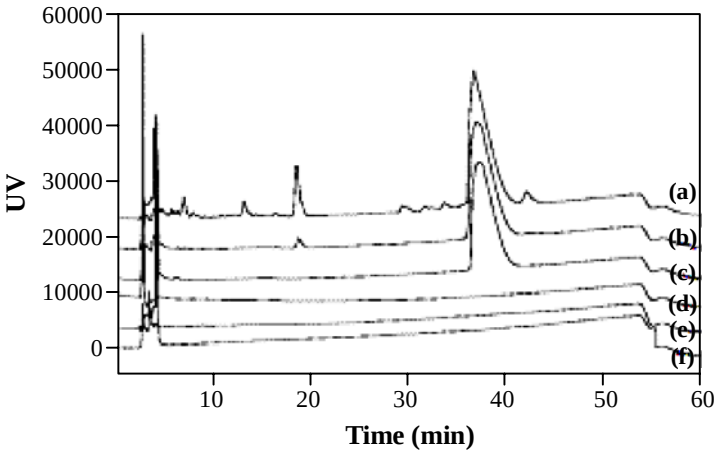

Fig. 19: Overlay of chromatograms from specificity study (a) System suitability solution (b) Fondaparinux injection (Arixtra) (c) Fondaparinux API (d) Blank –solution B (e) Blank – solution A (f) Blank – diluents

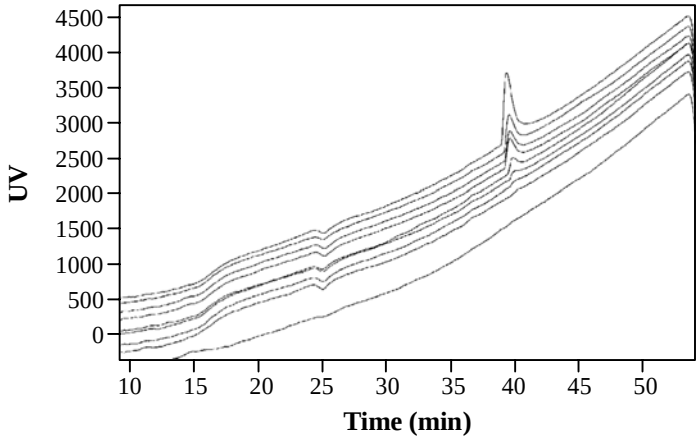

Fig. 20: Overlay of LOD chromatograms of Fondaparinux at concentration ranging from 0.0025 mg/mL to 0.1 mg/mL

A sample solution containing Fondaparinux at the LOD level was prepared and analyzed in replicates and the % RSD of the peak responses was found to be 3.05%, which

was within the acceptance criteria of NMT 15%. A sample solution containing Fondaparinux around its LOQ level concentration was prepared and analyzed to establish precision and accuracy at LOQ. The % RSD value obtained for the area of Fondaparinux at LOQ was 4.59%, which was within the acceptance criteria of NMT 15%. The percent recovery at the LOQ level was 79.11%, which was within the acceptance criteria of 70.0-130.0% of the theoretical concentration at the LOQ level.

Robustness

The method robustness measures the ability of the analytical method to tolerate minor variations in the method recommended parameters, demonstrating the reliability of the method under normal use. In order to demonstrate robustness a few of the parameters such as column temperature, mobile phase pH, and flow rate was varied intentionally and the system suitability parameters were evaluated with each variation. The system suitability criteria was met with all the variations; the method was found to be robust with variations of ± 5^{o}C in the column temperature, robust with variations in pH of the mobile phase (both solution-A and solution-B were evaluated individually) and robust with ± 0.2 mL/min flow variations in the mobile phase.

Solution stability

One of the standard and sample preparations at 100% level of the method concentration was stored at room temperature and was analyzed periodically to establish the solution stability. The response of analyte in the standard preparation and the content of the impurities in the sample solution were measured to establish the solution stability. The evaluations of the results obtained revealed that the standard solution and sample solutions are stable for a minimum period of 3 days when stored at room temperature.

CONCLUSION

The optimized pH gradient SAX-HPLC method using in situ lithium perchlorate mobile phase is a simple and high-sensitive analytical method for the determination of assay and chromatographic purity of Fondaparinux sodium in the formulation (Arixtra injection). This method employs an appropriate pH gradient mobile phase and a suitable stationary phase to increase the sensitivity of the method and to improve the resolution between the analytes. The insitu preparation of lithium perchlorate reduces the high drift in the baseline and makes the method more sensitive, reliable and cost-effective. The results of the validation study shows that this method is robust and can be used for the determination of assay and impurities in Fondaparinux sodium injection.

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