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RESEARCH ARTICLE

Analytical Method Development and Validation of HPLC for Identification and Assay of N-2 Intermediate of a Therapeutic Pentasaccharide- Fonda Parinux

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ABSTRACT

A simple, accurate Reverse Phase High Performance Liquid Chromatographic (RP-HPLC) method with UV detector (210 nm) was developed and validated for identification, determination of assay and purity of *Fonda-parinux* intermediate (N-2) content in In-Process. The best separation was achieved with octadecylsilane column diameter (250*4.6 mm; 5 µ) by using a gradient mobile phase 10 mM potassium di-hydrogen phosphate (pH = 6.0 adjusted with 1 M potassium hydroxide solution) and acetonitrile in the ratio of 70:30. The mobile phase were prepared and pumped at a flow rate of 1 ml/min. The above method is validated using the following parameters: Specificity, linearity, accuracy, precision, LOD and LOQ as per the ICH-Q2 (R1) guidelines.

KEYWORDS

Fonda Parinux, Octadecylsilane column, HPLC, Buffers, UV Detection

INTRODUCTION[1-4, 8-10]

In the early 1980's, a unique, linear, octasulfated pentasaccharide domain of heparin chains was discovered, responsible for the interaction between heparin and Anti-Thrombotic III. Twenty years later around 2002, a novel Anti-Thrombotic agent was launched, *Fonda-Parinux* that blocks factor Xa in the coagulation cascade. This sulfated low molecular weight heparin like molecule is an unbranched pentasaccharide constructed from the repetition of pyranosyluronic acid (either D-glucoronic or L-iduronic) and 2-amino-2-deoxyglucopyranose units. *Fonda-Parinux* is a therapeutic drug belonging to the group of the anti-thrombotic agents which are used to prevent deep vein thrombosis in patients undergoing orthopedic surgery. *Fonda-Parinux* is a synthetic sulfated pentasaccharide which acts as a selective indirect inhibitor of factor Xa. The Anti-Thrombotic activity of *Fonda-Parinux* is the result of Anti-Thrombotic (AT) III mediated selective inhibition of factor Xa. Binding selectively to Anti-Thrombotic III, *Fonda-Parinux* potentiates the innate neutralization of factor Xa by Anti-Thrombotic III. Neutralization of factor Xa interrupts the blood coagulation cascade and inhibits both thrombin formation and thrombus development. This low molecular weight heparins are either extracted from natural animal sources (porcine intestine or bovine lung) or degraded forms of such extractions.

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Structure of Fonda-Parinux

Fonda Parinux is a linear sulfated pentasaccharide (octasaccharide with five

monosaccharides units) molecule having five sulfate esters on oxygen and three sulfates on nitrogen. In addition, it contains five hydroxyl groups in the molecule that are not sulfated and two sodium carboxylates. Out of five derivatives, there are three glucosamine derivatives and one glucoronic acid and one L-iduronic acid. The five pentasaccharides are connected to each other in alternate α and β glycosylated linkages. Chemical structure is shown in figure 1.

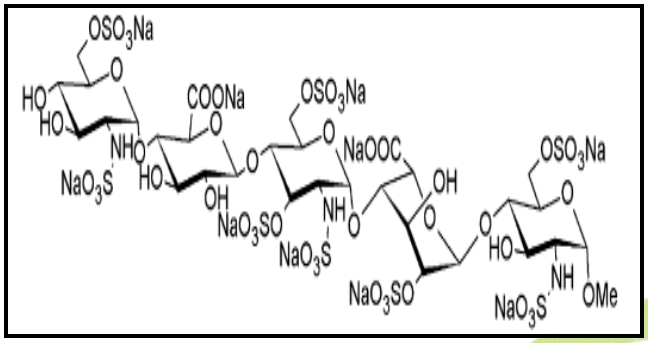

Figure 1: Structure of Fonda parinux

Properties of Fonda Parinux

Scientific Names: Fonda parinux sodium, Arixtra, Quixidar, Fonda parin sodium.

Empirical Formula: $C_{31}H_{43}N_3Na_{10}O_{49}S_8$.

Molecular Weight: 1726.77 g/mol or daltons.

pH: In between 5.0-8.0.

Solubility: It is dissolved in isotonic solution of Sodium Chloride (NaCl) & water. It is also soluble in Sodium Hydroxide (NaoH) and insoluble in ethanol.

Nature: It is a white to almost white hygroscopic powder. The final product is a clear & colorless to slightly yellow liquid.

Synthesis of Fonda Parinux[1-4]

Synthesis of *Fonda-parinux* requires four common transformation steps which are shown in figure 2.

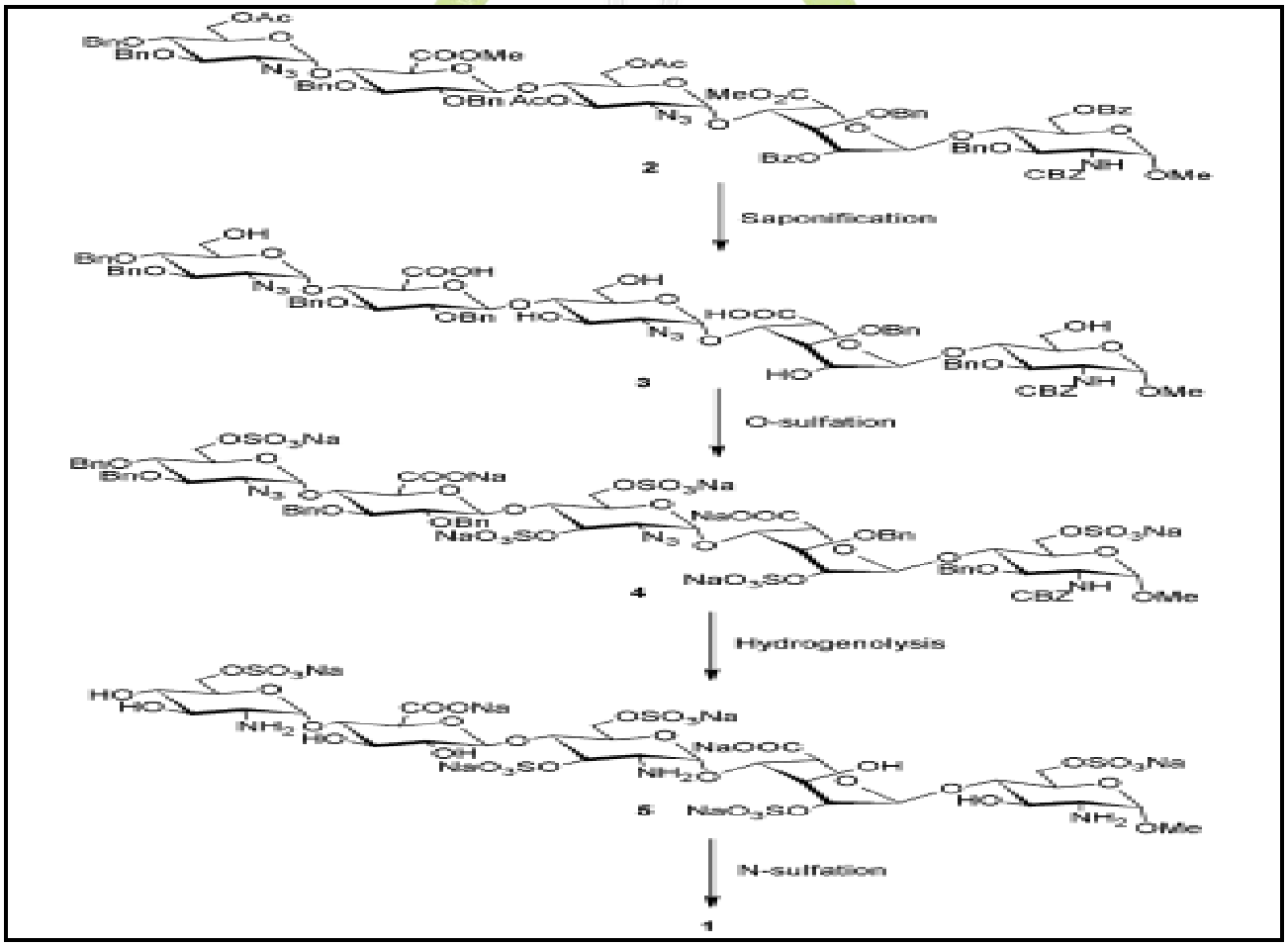

Figure 2: Synthesis of Fonda Parinux

Saponification: The Hydrolysis of acetyl, benzoyl, methyl ester groups leading to free hydroxyl groups being sulfated first. These esters were saponified by two step procedure using Lithium Hydroxide and Hydrogen peroxide in Tetrahydrofuran (THF), and then Sodium hydroxide in methanol.

O-Sulfation: O-sulfation under anhydrous conditions enabling the installation of sulfate moieties. The produced hydroxyl groups were further sulfated with triethylamine-sulfur trioxide complex in N,dimethylformamide to give pentasaccharide.

Hydrogenolysis: Hydrogenolysis of azide/NHCbz groups resulting in free amino functionalities with the simultaneous removal of benzyl ether groups, and hydrogen labile groups from protected hydroxyls. It is achieved by catalytical hydrogenation in the presence of Pd/C.

N-Sulfation: The selective sulfation of unmasking amino groups in the presence of unprotected hydroxyls gives the desired pentasaccharide.

The aim of this research work is to develop and validate a simple, accurate and specific reversed phase high performance liquid chromatographic method for identification, assay and impurities of *Fonda-parinux* intermediate (N-2) content in In-Process. Analysis of the drug was performed on Octadecylsilane column in a gradient mode employing 10 mM potassium di-hydrogen phosphate (pH = 6.0 adjusted with 1 M potassium hydroxide solution) and acetonitrile as the mobile phase in the ratio of 70:30 v/v.

The mobile phases were pumped at a flow rate of 1ml/min with UV detector at 210 nm. This method was validated by considering precision, accuracy, linearity and specificity to ensure the compliance in accordance with the ICH Q2 R1guidelines.

MATERIAL AND METHODS

Chemicals[7]

10 mMPotassium Di-hydrogen Phosphate, 1 M Potassium Hydroxide solution, 10 mM Di-Sodium Hydrogen Phosphate, 1 M Sodium Di-Hydrogen Phosphate, Acetonitrile (HPLC grade), Water for injection (WFI). All the buffers were purchased from Merck pvt Ltd, India.

Instrumentation

To develop a high performance liquid chromatography method for determining the assay & purity of Fonda parinux, a Dionex ultimate 3000 separation model HPLC equipped with UV detector, operated with chromeleon software and Octadecylsilane column with diameter (250 * 4.6 mm, 5 µ) were used along with Analytical Weighing balance, pH meter, Vacuum pump, Sonicator, Microwave oven.

Mobile Phase Preparation

Mobile phase A- Weigh 0.68 grams of potassium di-hydrogen phosphate and dissolve it in 300 ml of WFI. Adjust the pH at 6.0 with Potassium hydroxide. Make up the volume to 500 ml.

Preparation of Potassium hydroxide: Weigh 561.1 mg of Potassium hydroxide and dissolve it in 10 ml of WFI.

Mobile phase B- 100% Acetonitrile.

All the mobile phases were filtered using 0.2µ membrane filter and degassed using sonicator for 15-20min.

HPLC Separations

The High performance liquid chromatography separations of *Fonda parinux* was performed on a 5µm octadecylsilane column (250 * 4.6 mm) using Dionex. The gradient elution condition in the ratio of 70:30 was applied for equilibrating the column. This HPLC system consists of Solvent Reservoir, quaternary pumps, purge valve, injector, degasser, and UV detector. The eluents were monitored at 210 nm.

Standard Preparation

N-3: Weigh accurately about 1 mg of N-3 Working standard and dissolve it in 80% of Acetonitrile and 20% of WFI in the ratio 4:1 respectively. The sample concentration is 1 mg/ml.

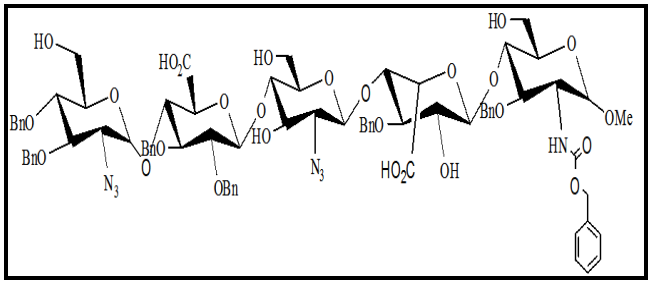

Figure 3: N-3 Sample

Sample Preparation

N-2 Sample: Weigh accurately about 5 mg of N-2 sample and dissolve it in 1 ml of WFI. The sample concentration is 5 mg/ml.

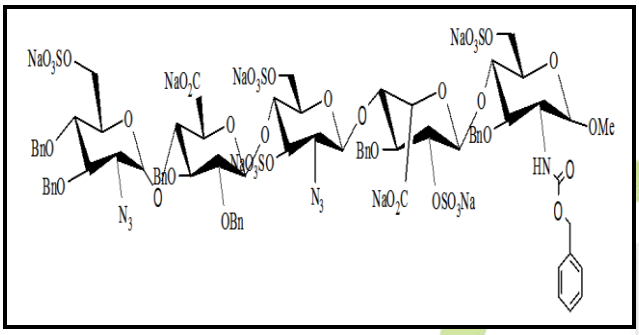

Figure 4: N-2 Sample

Table 1: Instruments used for the HPLC method

S. NO	Equipments and Instruments used	Manufacturer
1	HPLC	Thermoscientific/Dionex Ultimate 3000
2	Weighing Balance	Essae
3	pH meter	Polmon
4	Conductivity meter	Polmon
5	Vacuum pump	Tarsons ROCKWAVE 610
6	Sonicator	Fast Clean
7	Microwave Oven	Koryo
8	Glass wares	Borosil, Merck
9	Micropipettes	Thermoscientific
10	Eppendorf tubes	Tarsons
11	Gloves	RS SAFE
12	Vials	Chromatopak
13	Centrifuge tubes	Tarsons

RESULTS AND DISCUSSION

Method Development

All the chromatographic parameters were carefully analyzed & studied in order to recognize the most suitable chromatographic systems.

Type of Column

A Phenomenex Luna C18 column (250*4.6 mm), 5 µm particle size column was maintained at 30°C& gradient method was used for determining the assay & purity of Fonda parinux. Column oven temperature was also studied and it was found that good determination could be obtained at 30°C.

Mobile Phase Composition (Buffer-pH)

Choosing suitable mobile phase pH was an important factor. Two types of buffer (Potassium di-hydrogen phosphate & Di-Sodium hydrogen phosphate) were examined during development and analysis. It was found that potassium di-hydrogen phosphate buffer gave the best resolution with impurities, component selectivity and peak symmetry.

Choice of Flow Rate

The effect of flow rate was studied to optimize the chromatographic efficiency of the proposed method and improve the resolution of the eluted peaks. The flow rate of 1 ml/min was found to be

optimum for good separation in reasonable time. So, the best chromatographic performances were achieved when using gradient mobile phase composed of Acetonitrile & Potassium di-hydrogen phosphate, pH adjusted to 6.0, injection volume 100 µl, column temperature 30 degrees, detection wavelength 210 nm, flow rate 1 ml/min. Under the optimized conditions, Fonda samples were separated in between 16-18 minutes.

Detection Wavelength

HPLC analysis showed that Fonda-parinux has maximum absorbance at 210 nm. Therefore the chromatographic detection was performed at 210 nm using UV-Vis detector.

Table 2: Gradient program

Time (min.)	Flow Rate (ml/min)	Percentage of A (%)	Percentage of B (%)
0	1	70	30
10	1	60	40
25	1	30	70
30	1	30	70
31	1	70	30
36	1	70	30

Table 3: Chromatographic conditions for the proposed RP-HPLC method

S. No	Parameters	Conditions
1	Column	Phenomenex Luna C18 Octadecylsilane Column (250*4.6 mm),5 µ
2	Mobile phase A	10 mM Potassium di-hydrogen phosphate
	Mobile phase B	100% Acetonitrile
3	UV detection	210 nm
4	Flow rate	1ml/min
5	Injection volume	N-2 (20 µl)
6	Temperature	30 degrees
7	Retention time	16-17 minutes
8	Run Time	36 minutes
9	Analytical HPLC	DIONEX ULTIMATE 3000

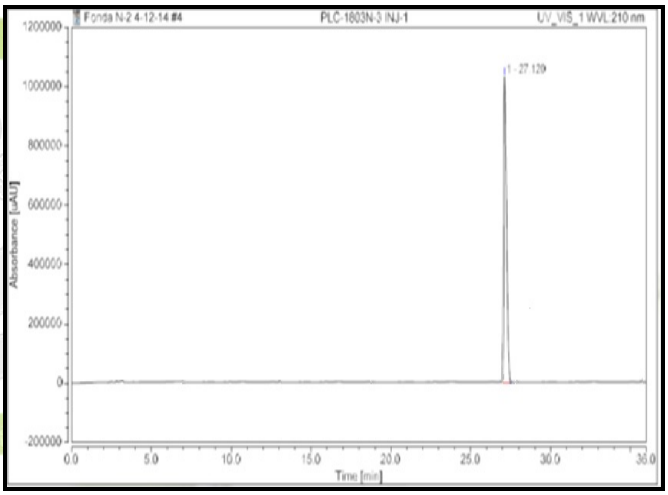

Figure 6: Chromatogram of N-3

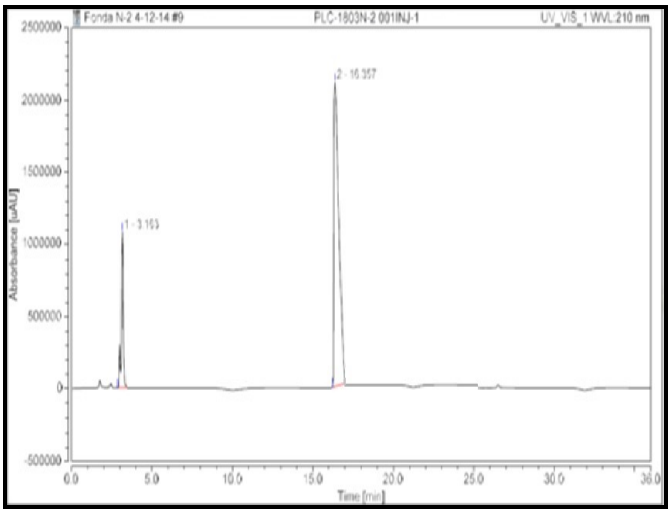

Figure 7: Chromatogram for N-2 Intermediate

Validation[5-6]

The method was validated for System Suitability, Accuracy, Precision, Linearity, LOQ and LOD according to the International Conference on Harmonization ICH guidelines (Q2-R1).

System Suitability

System suitability testing is an integral part of many analytical procedures. System suitability was assessed by injecting six injections of the Fonda Parinux solution into the HPLC system and the chromatogram with the area response was obtained. The system suitability parameters such as Peak Area, Tailing factor, Theoretical Plates, % RSD (Relative Standard Deviation) and area of the six replicates were calculated from the chromatogram.

Table 4: Observations for System Suitability

S. No	Peak Area (AU)	Tailing Factor	Theoretical Plates
System suitability 1	313.809	1.07	76460
System suitability 2	313.623	1.06	76820
System suitability 3	313.159	1.84	76328
System suitability 4	313.85	1.81	76881
System suitability 5	313.923	1.04	76665
System suitability 6	313.625	1.021	76328
Standard Deviation	0.276016		
Standard mean	313.6648		
% RSD	0.087997		
Standard mean 2	313.809		
Recovery	99.95379355		

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. Typically these might include impurities, degradants, and matrix, etc. We did not observe any interfering peaks which co-eluted with the compound interest. The retention times of the N-2 and N-3 intermediates were approximately 16.0 min and 27.0 min respectively. Hence the method is specific and selective for N-2 intermediate.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Here the accuracy was carried out in triplicate. The accuracy was determined in terms of recovery at three concentrations 75%, 100%, 125%. From the result, recovery was found in the range of 99.84% to 100.01%, which indicated the accuracy of the method was adequate.

Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The precision was determined in-terms of Repeatability, Intermediate precision.

Repeatability

Repeatability should be assessed either using

a. a minimum of 9 determinations covering the specified range for the procedure.

b. a minimum of 6 determinations at 100% of the test concentration.

Six determinations were carried out at 100% of the test concentrations. The Average, Standard deviation and % Relative Standard Deviation were calculated and found to be 313.6375, 0.22319655 and0.07116386 respectively.

Table 5: Observations for Accuracy

S.No	Sample Area	Standard Area	% Recovery
75% Spiked 1	235.356	235.731	99.84092037
75% Spiked 2	235.512	235.731	99.9070975
75% Spiked 3	235.345	235.731	99.83625404
100% Spiked 1	313.859	313.812	100.0149771
100% Spiked 2	313.732	313.812	99.97450703
100% Spiked 3	313.658	313.812	99.95092603
125% Spiked 1	392.984	392.965	100.004835
125% Spiked 2	392.816	392.965	99.96208314
125% Spiked 3	392.924	392.965	99.9895665

S.No	75%	100%	150%
Standard	235.731	313.812	392.965
Spiked 1	235.356	313.859	392.984
Spiked 2	235.512	313.732	392.816
Spiked 3	235.345	313.658	392.924
Standard Deviation	0.180260922	0.088661811	0.075128224
AVG	235.486	313.76525	392.92225
% RSD	0.076548467	0.028257371	0.019120379

Table 6: Observations for Repeatability

Repeatability	
Run	**Area**
100% Nominal 1	313.854
100% Nominal 2	313.821
100% Nominal 3	313.726
100% Nominal 4	313.564
100% Nominal 5	313.616
100% Nominal 6	313.244
Standard Deviation	0.22319655
Average	313.6375
% RSD	0.07116386

Intermediate Precision

Intermediate precision refers to variations within a laboratory as with different days, with different instruments by different analysts, and so forth. Six determinations were carried out at 100% of the test concentrations. The Mean, Standard deviation and % Relative Standard Deviation were calculated and found to be 313.543, 0.242706407 and 0.077407694 respectively.

Table 7: Observations for Intermediate Precision

Intermediate Precision	
Run	**Area**
100% Nominal 1	313.624
100% Nominal 2	313.621

100% Nominal 3	313.809
100% Nominal 4	313.664
100% Nominal 5	313.116
100% Nominal 6	313.424
STD Deviation	0.242706407
Mean	313.543
% RSD	0.077407694

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. The calibration plot was found to be linear in the range of 50-150 µg mL^{-1}. Linearity of fondaparinux was plotted by a graph of mean area versus concentration. The Slope of the graph (m), the correlation co-efficient (r), Y-Intercept (c) were calculated and found to be 3.1564, 0.9999, -1.7736 respectively.

Table 8: Observations for Linearity

Concⁿ	**50%**	**75%**	**100%**	**125%**	**150%**
Nominal 1	156.904	234.465	313.216	392.162	472.713
Nominal 2	157.12	235.812	313.546	391.284	472.784
Nominal 3	156.931	232.524	313.809	391.986	472.812
Avg.	156.985	234.267	313.523	391.81	472.769
Stand. Deviation			0.29713		

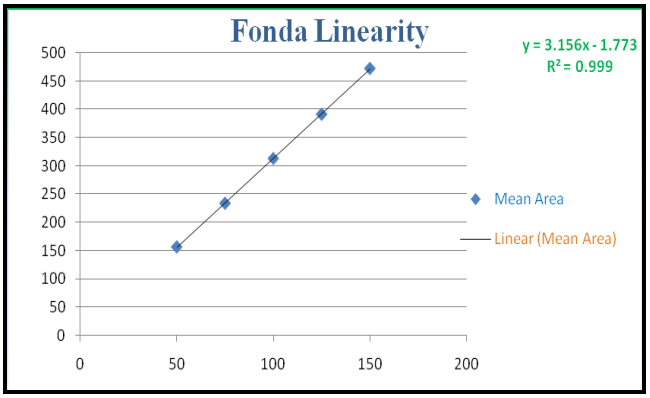

Figure 8: Graph for Fonda Parinux Linearity

Table 9: Calibration plot data for Fonda Parinux

S.No	**Concentration (µg mL^{-1})**	**Mean Peak area**
1	50	156.985
2	75	234.267
3	100	313.523
4	125	391.81
5	150	472.769
6	**Correlation coefficient (r)**	0.9999
7	**Slope (m)**	3.1564
8	**Y Intercept (c)**	-1.7736

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Limit of detection is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value, whereas Limit of Quantification is lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The LOD and LOQ were calculated from the linearity of Fonda Parinux. The LOD and LOQ were found to be 0.31064 µg mL^{-1} and 0.94135 µg mL^{-1} respectively.

Table 10: Observations of LOD and LOQ

S.No	Parameters	Formula	Results (µg mL^{-1})
1	LOD	3.3 * Standard Deviation / slope	0.31064
2	LOQ	10 * Standard Deviation / slope	0.94135

CONCLUSION

The Reverse Phased High Performance Liquid Chromatography (RP-HPLC) method was developed and validated for identification and assay of *Fonda Parinux* intermediate (N-2) content as per the standard parameters given in the validation protocol. The method shows a good performance with respect to system suitability, accuracy, precision, linearity, LOD and LOQ as per the proposed limits given in validation protocol. Finally, by considering the summarized data, it is concluded that method can be easily and conveniently used for the analysis of *Fonda Parinux* intermediate (N-2).

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REFERENCES

1. http://en.wikipedia.org/wiki/Fondaparinux.
2. Kozioł, A., Lendzion-Paluch, A., & Manikowski, A. (2013). A Fast and Effective Hydrogenation Process of Protected Pentasaccharide: A Key Step in the Synthesis of Fondaparinux Sodium. *Organic Process Research & Development*, *17*(5), 869-875.
3. Walenga, J. M., Fareed, J., Jeske, W. P., Frapaise, F. X., Bick, R. L., & Samama, M. M. (2002). Development of a synthetic heparin pentasaccharide: fondaparinux. *Turkish Journal of Hematology*, *19*(2), 137-150.
4. Li, T., Ye, H., Cao, X., Wang, J., Liu, Y., Zhou, L., & Wang, P. (2014). Total Synthesis of Anticoagulant Pentasaccharide Fondaparinux. *ChemMedChem*, *9*(5), 1071-1080.
5. ICH Topic Guideline, I. H. T. (2005). Validation of analytical procedures: text and methodology. *Q2 (R1)*, *1*.
6. Guideline, I. H. T. (2005). Validation of analytical procedures: text and methodology. *Q2 (R1)*, *1*.
7. The Merck Index. Fourteenth Edition, page no's: 13, 14, 44, 45, 50, 53.
8. Nadji, Sourena, James, T. S., Joseph A. Vanartsdalen, (2011), Patent no. WO2011014793 A2.
9. Payal Parthpatel, Chun Ma, Kevin K. Ohrr, SourenaNadji, (2013), Patent no. Us8420790 B2.
10. Shang-Cheng Hung, Taipei City 116 (TW), Cheng–Hsiu Chang, Changua City (TW), (2015), Patent no. US 2015/0126721 A1.

