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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF PLERIXAFOR AND RELATED SUBSTANCES IN BULK FORM.

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ABSTRACT

A reverse phased HPLC method for the estimation of related substance present in Plerixafor in bulk form has been validated as per ICH guidelines. The method was developed using C18 (Kromosil 250x4.6 mm,5µm) column using the gradient program with mobile phase A (Buffer and Acetonitrile 80:20) and mobile phase B (Buffer and Acetonitrile 20:80) pH maintained at 3±0.05 and were monitored at 215 nm. The linearity level for Plerixafor was established with concentration of drug extending from 0.010 to 0.201%. The linearity level for Plerixafor impurity (H-PXFRC01 & H-PXFRC02) was established with concentration ranging from 0.005 to 0.300% & 0.007 to 0.301%. The % recovery obtained between 99.1 to 99.5% & 105.4 to 106.0%, proved that the method was accurate. The system precision results were within the limits. The low values of RSD showed that the method is precise. LOD and LOQ of method were determined, based on signal to noise method. Robustness of the proposed method was ascertained by deliberately changing the pH variation, flow rate of mobile phase and mobile phase stability. There was no significant change in the system suitability factors of Plerixafor and its impurity peak when these parameters were changed. The low values of the % RSD indicated the robustness of the method.

KEYWORDS: HPLC method validation, Plerixafor, Acetonitrile.

INTRODUCTION

Plerixafor is an anticancer drug,[1] it stimulates the release of stem cells from the bone marrow into the blood in patients with non-Hodgkin lymphoma and multiple myeloma.[2] These stem cells are then collected and used in autologous stem cell transplantation to replace blood-forming cells that were destroyed by chemotherapy.[3] Plerixafor has orphan drug

status in the United States and European Union; it was approved by the U.S. Food and Drug Administration on December 15, 2008. Plerixafor inhibits the CXCR4 chemokine receptor and reversibly blocks binding to the marrow compartment of its cognate ligand, stromal cell derived factor-1-alpha (SDF-1alpha), which play a role in the trafficking and homing of human hematopoietic stem cells.[4,9] Phase III studies have demonstrated that plerixafor combined with granulocyte-colony stimulating factor (G-CSF) improves PBSC collection compared to mobilization with G-CSF alone in patients with MM or NHL.[10,11] Phase II study was undertaken to investigate the efficacy and safety of hematopoietic stem cell mobilization with plerixafor in patients with HL.[12] Plerixafor[13] is chemically 1,1'-[1,4-phenylenebis(methylene)]bis[1,4,8, 11- tetraazacyclotetradecane]. (Figure No. 1).

The objective of this work was to present a stability-indicating method to estimate Plerixafor and its related compounds. In the present work, a simple, fast and precise liquid chromatographic method was developed for the determination of Plerixafor and its impurities.

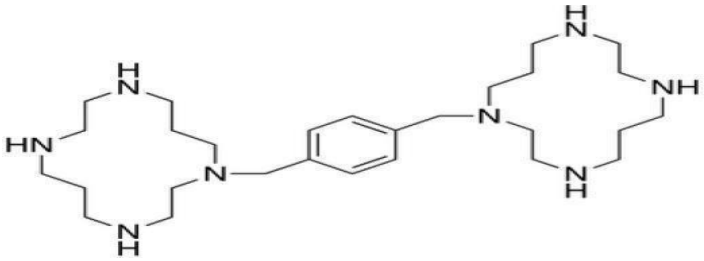

Figure No. 1: Structure of Plerixafor.

MATERIALS AND METHODS

Chemicals and reagents

The Plerixafor standard and its impurities were supplied by Aurbindo pharma Pvt Ltd, Mumbai, India. The HPLC-grade Acetonitrile, sodium-1-heptanoicsulfonic acid, Perchloric acid and Sodium hydroxide were purchased from Merck. HPLC-grade water was prepared using the Millipore Milli-Q Plus, water purification system.

Chromatographic conditions and equipment

The LC system of Agilent LC 1200, Waters 2695 pump with a PDA detector was used for this study and chromatographic separation was achieved on the C_{18} Kromosil RP (250x 4.6 mm, 5µm) column as the stationary phase. The separation was achieved by the gradient method.

Mobile phase A contained buffer and acetonitrile in the ratio of 80:20 (v/v). Mobile phase B contained a mixture of buffer and acetonitrile in a ratio of 20:80 (v/v). The HPLC gradient program (T/%B) was set as 0.01/10, 4.0/20, 40.0/50, 45.0/10 and 55.0/10 with the flow rate of 1.0 mL/min. The peaks were monitored at the wavelength of 215 nm, keeping the column temperature at 30^0C using injection volume of 20 µL.

Preparation of Mobile phase, Standard solution and Sample solution

For preparations of buffer solution, dissolved about 4.32 g of sodium-1-hepatnoic sulphonic acid in 1000 mL of water, added 5.0 mL of Perchloric acid and mixed. Adjusted the pH of this solution to 3 ± 0.05 with 5 N sodium hydroxide solution. A mixture of Water and Acetonitrile in the ratio of 80:20v/v was used to get diluent. To get mobile phase-A, a mixture of buffer and Acetonitrile in the ratio 80:20%v/v was used. To get mobile phase-B, a mixture of buffer and Acetonitrile in the ratio 20:80%v/v was used. Filtered and degassed through 0.45µm membrane filter paper. Diluent used as blank solution. For reference solution, weighed accurately about 25mg of Plerixafor standard into a 100mL volumetric flask, dissolved and diluted to the volume with diluents and mixed. Diluted 1.0mL of this solution to 100mL with diluent and mixed.

Method development

The main target of the chromatographic method is to achieve the separation of impurities and the main component Plerixafor with each other. A blended solution containing 500 mg/mL of Plerixafor and 5 mg/mL of each impurity was prepared in diluent used for the method's development.

Method validation

The developed analytical method was validated for its acceptable performance to ensure suitability of indent purpose. The validation parameters like accuracy, precision, specificity, detection limit, quantification limit, linearity, range, ruggedness and robustness experiments were executed. The proposed method was validated as per ICH guidelines.[14,15]

RESULTS

Method development

For the optimised chromatogram the retention time and area are summarized in Table I, Figure No 2.

Table 1: Optimized results.

S. No	Name	Retention time	Area
1	H-PXFRC01	6.669	2377
2	H-PXFRC02	10.381	1825
3	Plerixafor	16.411	39235113

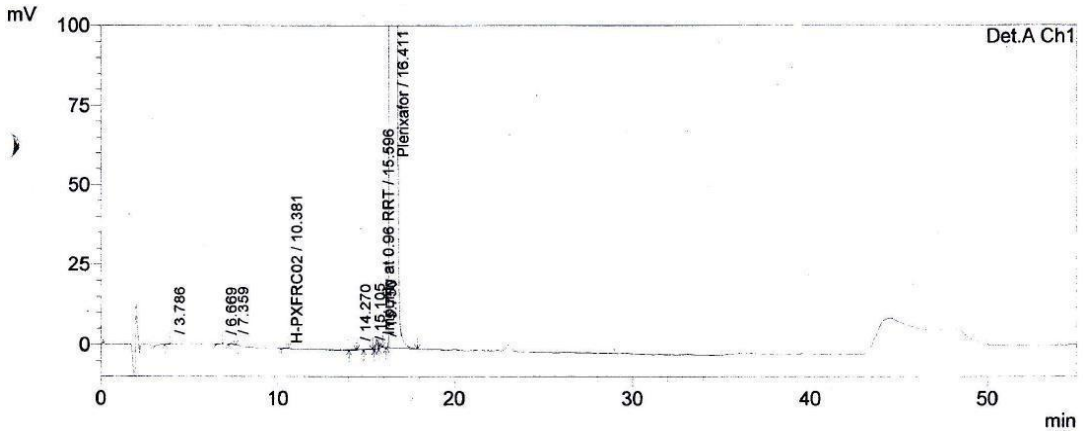

Figure No. 2: Optimized chromatogram of sample.

Method validation

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of all its potential impurities. No interference was observed due to blank at the retention time of H-PXFRC01, H-PXFRC02 and Plerixafor. The elution order & the relative retention times obtained from individual solution & blend solution were matched. Peak purity passed for H-PXFRC01, H-PXFRC02 and Plerixafor from individual solution and H-PXFRC01, H-PXFRC02 form blend solution. The specificity (Figure No.3) of the method was demonstrated by analysing blank and impurity in the test solution, and is found to be free from interferences.

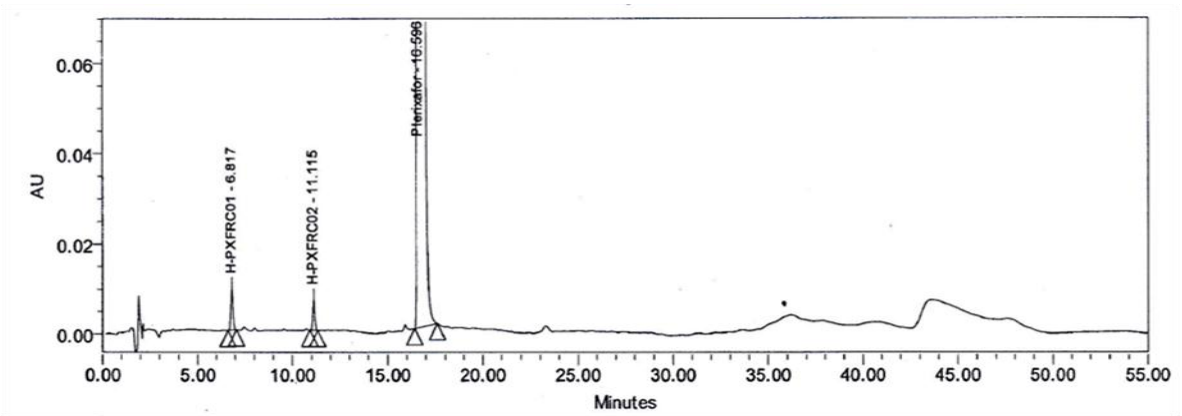

Figure No. 3: Blend solution chromatogram.

Limit of detection (LOD) and Limit of quantification (LOQ)

Signal-to-Noise ratio of Plerixafor and its impurity (H-PXFRC01) (H-PXFRC02) were found as 4.6, 4.2 & 3.4 respectively for LOD(Figure no 4). Signal-to-Noise ratio of Plerixafor and its impurity (H-PXFRC01) (H-PXFRC02) were found as 12.4, 11.3 and 11.0 respectively for LOQ(Table 2, Figure no 5).

Table 2: LOD and LOQ Values.

		LOD		LOQ	
S. No.	**Name**	**DL (in%) w.r.t test sample concentration**	**S/N ratio**	**DL (in%) w.r.t test sample concentration**	**S/N ratio**
1	H-PXFRC01	0.002	4.6	0.005	12.4
2	H-PXFRC02	0.003	4.2	0.007	11.3
3	Plerixafor	0.004	3.4	0.01	11.0

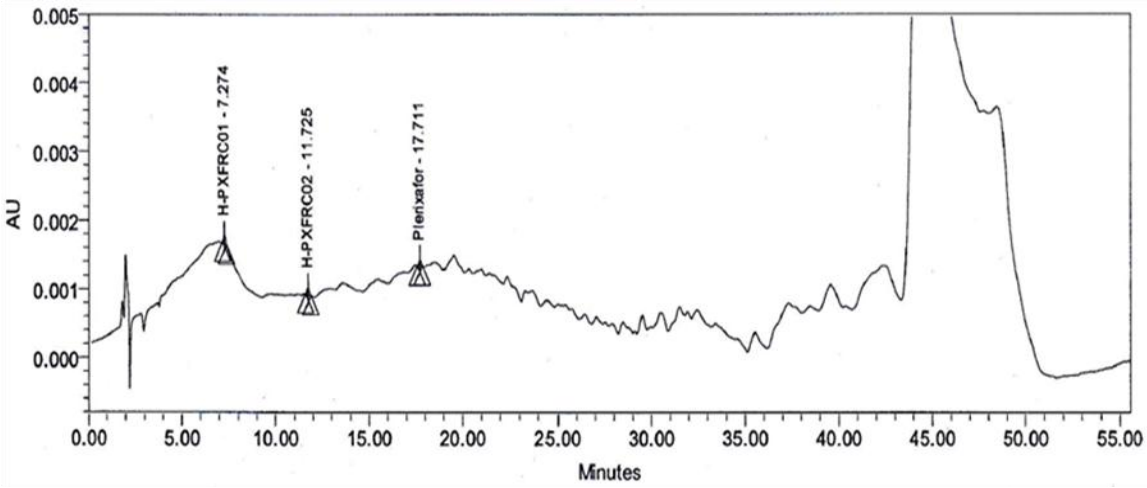

Figure No. 4: LOD solution chromatogram.

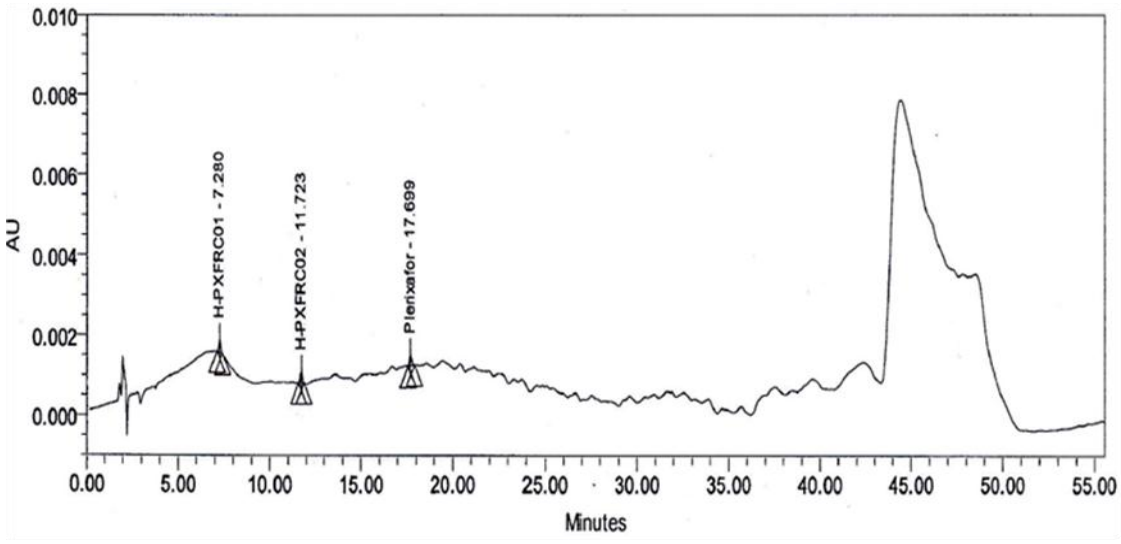

Figure No. 5: LOQ solution chromatogram.

Precision

The %RSD Recovery of each component shown in Table 3 were in the range of 0.96 to 4.60 and the acceptance limit is, %RSD Recovery of each component is should not me more than 10.

Table 3: Precision.

Injection No.	Peak area counts		
	H-PXFRC01	H-PXFRC02	Plerixafor
1	2558	2246	2241
2	2550	2071	2204
3	2523	2101	2304
4	2589	2296	2330
5	2547	2122	2384
6	2524	2289	2304
Average	**2549**	**2188**	**2295**
%RSD	**0.96**	**4.60**	**2.79**

Accuracy

The accuracy of the method was determined by performing the recovery experiment of impurities at 3 levels (50%, 100%, and 150%). The % recovery obtained should be between 85.0% and 115.0%. The results shown in table 4 for H-PXFRC01 and table 5 for H-PXFRC02.

Table 4: Recovary of H-PXFRC01.

Sample No.	Spike level	% of impurity added	% of impurity found (recovered)	% Recovery	Average %recovery	%RSD
1	50%	0.0750	0.0753	100.4	99.9	0.56
2			0.0750	100.0		
3			0.0745	99.3		
4	100%	0.1499	0.1460	97.4	98.6	1.08
5			0.1488	99.3		
6			0.1487	99.2		
7	150%	0.2249	0.2231	99.2	99.3	0.15
8			0.2234	99.3		
9			0.2238	99.5		

Table 5: Recovery of H-PXFRC02.

Sample No.	Spike level	% of impurity added	% of impurity found (recovered)	% Recovery	Average %recovery	%RSD
1	50%	0.0750	0.0794	105.9	105.2	0.67
2			0.0784	104.5		
3			0.0788	105.1		
4	100%	0.1501	0.1623	108.1	106.3	1.61
5			0.1572	104.7		
6			0.1593	106.1		
7	150%	0.2251	0.2378	105.6	105.7	0.22
8			0.2376	105.6		
9			0.2385	106.0		

Linearity of Plerixafor

The linearity level for Plerixafor (Table 6, Figure 6) was established from QL level to linearity level-6, with concentration of drug extending from 0.010 to 0.201% with R^2 value of 0.9993. The linearity level for Plerixafor impurity (H-PXFRC01 & H-PXFRC02) shown in Table 7,8 and Figure 7,8 was established from QL level to linearity level-6, with concentration ranging from 0.005 to 0.300% & 0.007 to 0.301% with R^2value of 1.000 & 1.000. The calibration plot of peak area against concentration was linear in the range investigated and the linear regression data for the calibration plot are indicative of a good linear relationship between peak area and concentration over a wide range.

Table 6: Linearity of Plerixafor.

Level	Conc. (%)	Area of replicate-1	Area of replicate-2	Area of replicate-3	Average	%RSD
Level-1	0.010	2241	2204	2304	2250	2.25
Level-2	0.050	16114	16607	16653	16458	1.82
Level-3	0.080	24667	24529	23069	24088	3.68
Level-4	0.100	32086	32365	32055	32169	0.53
Level-5	0.151	49955	50555	50149	50220	0.61
Level-6	0.201	65908	66055	66075	66013	0.14

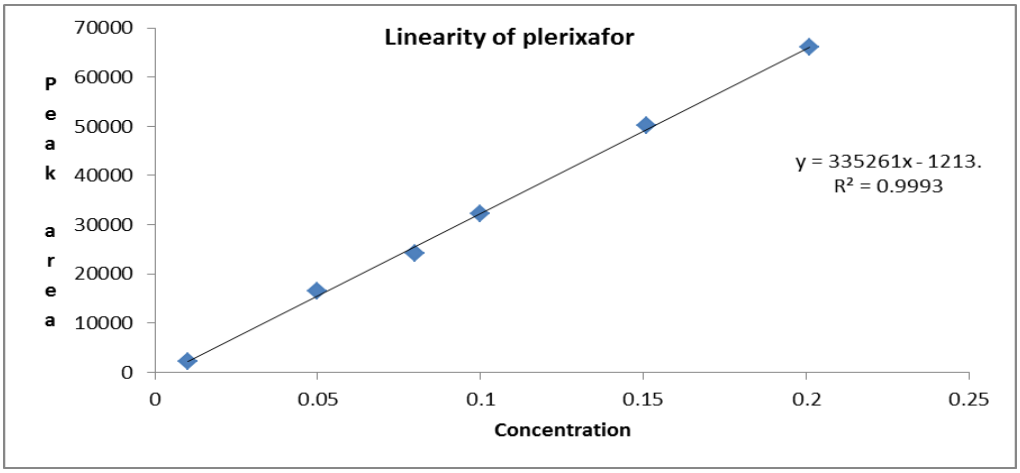

Figure No. 6: Linearity curve of Plerixafor.

Table 7: Linearity of H-PXFRC01.

Level	Conc. (%)	Area of replicate-1	Area of replicate-2	Area of replicate-3	Average	%RSD
Level-1	0.005	2558	2550	2523	2544	0.72
Level-2	0.075	38850	3889	38791	38827	0.08
Level-3	0.120	61825	61895	62048	61923	0.18
Level-4	0.150	77430	77638	77495	77521	0.14
Level-5	0.225	115466	115394	115480	115447	0.04
Level-6	0.300	153667	154309	154025	154000	0.21

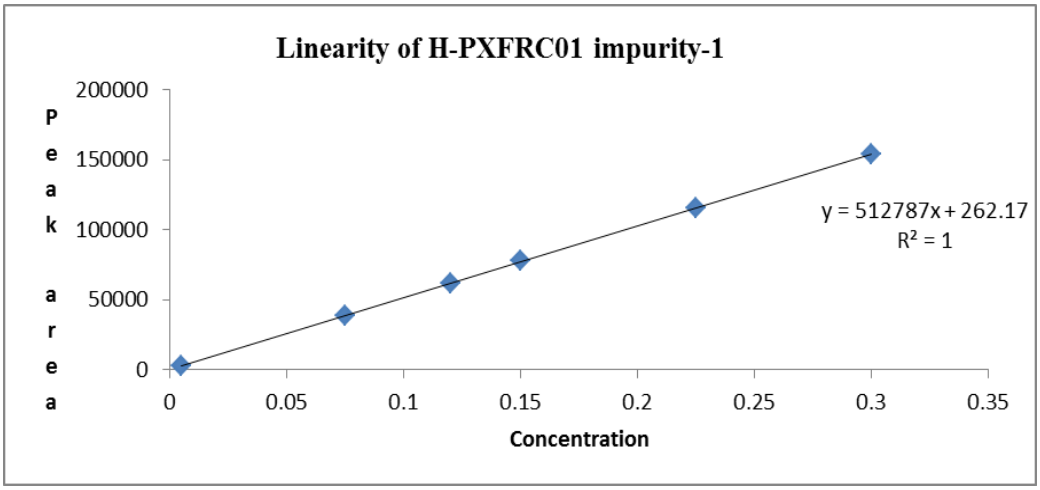

Figure No. 7: Linearity curve of impurity-1.

Table 8: Linearity of H-PXFRC02.

Level	Conc. (%)	Area of replicate-1	Area of replicate-2	Area of replicate-3	Average	%RSD
Level-1	0.005	2246	2071	2101	2139	4.38
Level-2	0.075	25031	25363	25139	25178	0.67
Level-3	0.120	40232	39694	39603	39843	0.85
Level-4	0.150	49474	49362	49737	49524	0.39
Level-5	0.226	74903	75479	75424	75269	0.42
Level-6	0.301	101172	100038	100205	100472	0.61

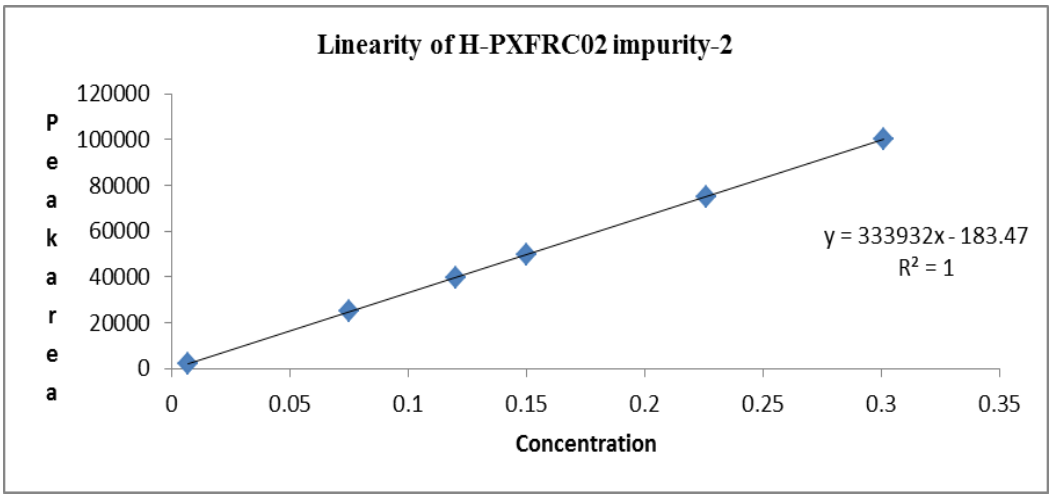

Figure No. 8: Linearity curve of impurity-2.

System Precision

Prepared and injected the reference solution in two replicate injections and calculated % RSD for the peak area response of each component obtained from two replicate injections of reference solution. Prepared and injected the reference solution in six times and calculated

%RSD. % RSD for peak Areas response of six replicate for Plerixafor and in standard solution was as found 0.58 shown in Table 9.

Table 9: System precision.

Injection No.	Peak area counts Plerixafor
1	29377
2	29382
3	29531
4	29080
5	29149
6	29209
Average	**29288**
% RSD	**0.58**

Method Precision

Performed the analysis by spiking the test sample with H-PXFRC01 & H- PXFRC02 at 100% level for six times and determined the method precision. Calculated the % of H-PXFRC01, H-PXFRC02, MSUI, and total impurities of each individual preparation as per the method. From six replicate injections, the % RSD for % impurity (H-PXFRC01), (H-PXFRC02), %MSUI and % of total impurities were found as 0.83, 1.1, 6.0 and 1.21 respectively shown in Table 10.

Table 10: Method Precision.

Injection No	Results in %			
	H-PXFRC01	**H-PXFRC02**	**MSUI**	**TI**
1	0.175	0.182	0.020	0.43
2	0.178	0.177	0.021	0.42
3	0.178	0.179	0.022	0.43
4	0.178	0.181	0.020	0.42
5	0.179	0.181	0.023	0.43
6	0.179	0.178	0.022	0.43
Average	**0.18**	**0.18**	**0.03**	**0.43**
%RSD	**0.83**	**1.11**	**6.00**	**1.21**

Intemediate Precision

Intermediate precision study was carried by performing study on a different day, with different instrument, different analyst and different column using fresh preparations**.** Prepared and injected reference solution in six replicate and recorded the chromatogram. Prepared and injected spiked solution in six preparation and calculated the % of H-PXFRC01, H-PXFRC02, MSUI and total impurities of each individual preparation as per the method. The

% RSD of % impurity (H-PXFRC01) (H-PXFRC02), %MSUI, % of TI from six replicate injections were found as 0.31, 0.94, 1.67 and 1.03 respectively shown in Table 11.

Table 11: Intermediate precision.

Injection No	Results in %			
	H-PXFRC01	H-PXFRC02	MSUI	TI
1	0.162	0.164	0.026	0.40
2	0.162	0.165	0.026	0.40
3	0.161	0.162	0.025	0.40
4	0.162	0.166	0.026	0.41
5	0.161	0.165	0.025	0.40
6	0.162	0.166	0.025	0.40
Average	**0.16**	**0.16**	**0.03**	**0.40**
%RSD	**0.31**	**0.94**	**1.67**	**1.03**

Test Solution state stability

Prepared and injected spiked test sample solution in duplicate (fresh sample preparations) and record the chromatogram. Injected each interval stability sample solution in duplicate and recorded the chromatograms. Compared the % variation of each H-PXFRC01, H-PXFRC02 obtained from initial sample (fresh sample) and each time interval of stability sample solution, reported the solution stability in hours. The variation of content (%) of H-PXFRC01 obtained from initial (fresh) sample and various hours study (12hours, 24 hours) concluded that the drug was stable up to 24 hours. The results shown in Table 12,13 and figure 9,10 respectively.

Table 12: After 12 hrs test solution state stability.

Name of the impurity	Peak area of relevant impurity in test solution	Initial preparations average content of impurity in % (Fresh)	After 12 hrs preparations average content of impurity in %	Variation
H-PXFRC01	75668	0.17	0.17	0.00
H-PXFRC02	51023	0.17	0.17	0.17
MSUI	8981	0.02	0.03	0.01
TI	-----	0.42	0.45	0.03

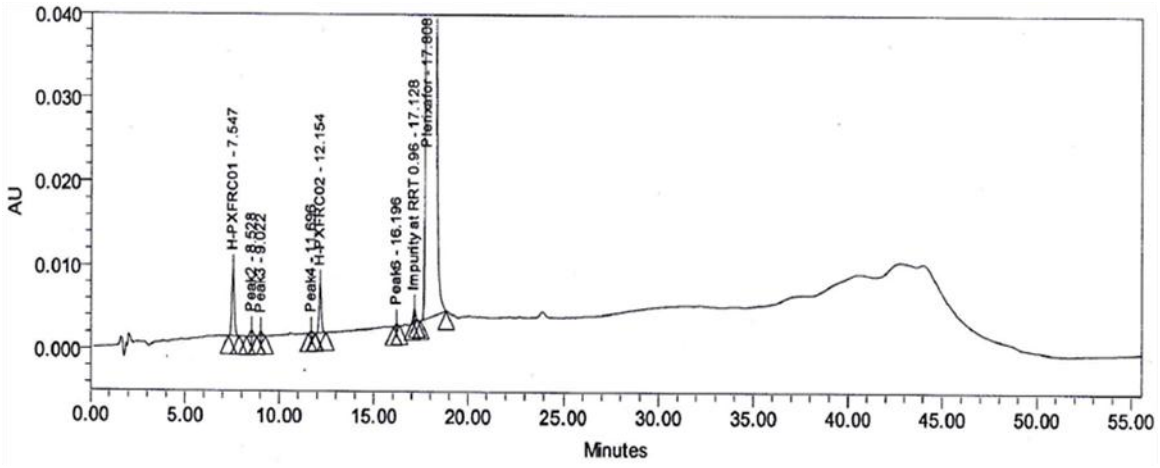

Figure No. 9: Test solution spiked with impurities after 12 hrs.

Table 13: After 24 hrs test solution state stability.

Name of the impurity	Peak area of relevant impurity in test solution	Initial preparations average content of impurity in % (Fresh)	After 24 hrs preparations average content of impurity in %	Variation
H-PXFRC01	75753	0.17	0.17	0.00
H-PXFRC02	50728	0.17	0.17	0.00
MSUI	9623	0.02	0.03	0.01
TI	------	0.42	0.46	0.04

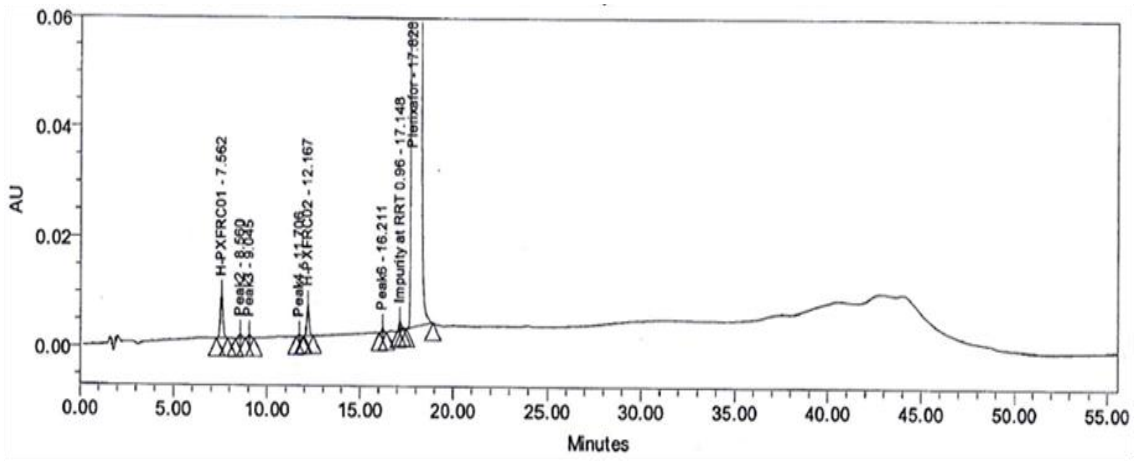

Figure No. 10: Test solution spiked with impurities after 24 hrs.

Robustness results

Robustness of the proposed method was ascertained by deliberately changing the pH variation, flow rate of mobile phase and mobile phase stability. There was no significant change in the system suitability factors of Plerixafor and its impurity peak when these parameters were changed. The low values of the % RSD indicated the robustness of the method. The results shown in Table 14-18.

Table 14: Robustness results for flow variation of 0.7mL/min.

Robustness	Result in %			
	H-PXFRC01	H-PXFRC02	MSUI	TI
Preparation-1	0.176	0.178	0.024	0.43
Preparation-2	0.177	0.179	0.024	0.43
Preparation-3	0.176	0.177	0.023	0.43
Average	**0.18**	**0.18**	**0.02**	**0.43**
% RSD	**0.33**	**0.56**	**3.00**	**0.00**

Table 15: Robustness results for flow variation of 0.9mL/min.

Robustness	Result in %			
	H-PXFRC01	H-PXFRC02	MSUI	TI
Preparation-1	0.180	0.185	0.025	0.43
Preparation-2	0.180	0.184	0.024	0.43
Preparation-3	0.180	0.185	0.025	0.43
Average	**0.18**	**0.18**	**0.02**	**0.43**
% RSD	**0.00**	**0.33**	**3.00**	**0.00**

Table 16: Robustness results for buffer variation 2.15.

Robustness	Result in %			
	H-PXFRC01	H-PXFRC02	MSUI	TI
Preparation-1	0.163	0.163	0.026	0.42
Preparation-2	0.163	0.163	0.025	0.41
Preparation-3	0.165	0.163	0.026	0.42
Average	**0.16**	**0.16**	**0.03**	**0.42**
% RSD	**0.75**	**0.00**	**2.00**	**1.38**

Table 17: Robustness results for buffer variation 2.25.

Robustness	Result in %			
	H-PXFRC01	H-PXFRC02	MSUI	TI
Preparation-1	0.165	0.161	0.023	0.40
Preparation-2	0.166	0.162	0.024	0.41
Preparation-3	0.165	0.160	0.023	0.41
Average	**0.17**	**0.16**	**0.02**	**0.41**
% RSD	**0.35**	**0.63**	**3.00**	**1.41**

Table 18: Robustness results for Mobile phase state stability.

Robustness	Result in %			
	H-PXFRC01	H-PXFRC02	MSUI	TI
Preparation-1	0.164	0.164	0.024	0.41
Preparation-2	0.164	0.164	0.024	0.41
Preparation-3	0.164	0.164	0.024	0.41
Average	**0.16**	**0.16**	**0.02**	**0.41**
% RSD	**0.00**	**0.00**	**0.00**	**0.00**

CONCLUSION

A simple gradient RP-HPLC method has been developed and validated for the determination of related substances of Plerixafor in bulk form. The developed method has been found to selective, sensitive, precise and robust. The method can be directly adopted in quality control laboratories for routine analysis with respect to determination of related substances of Plerixafor in bulk form.

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