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DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING RP-HPLC METHOD FOR PLERIXAFOR WITH ION-PAIR CHROMATOGRAPHIC APPROACH

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ABSTRACT

A novel, reversed-phase high-performance liquid chromatographic method was developed and validated for the Assay of Plerixafor (PLX) drug substance. PLX is an immune stimulant used to mobilize hematopoietic stem cells in cancer patients. The method was developed using Agilent Zorbax Eclipse plus C18 column using the isocratic program with mobile phase as a mixture of buffer and acetonitrile (65:35) v/v. PLX is monitored at 210 nm. The present work is describing the role of ion-pair reagent in the separation of polar compounds. PLX was subjected to various stress conditions of oxidative, acid, base, hydrolytic, thermal, humidity and photolytic degradations. The degradation products were well separated from each other and with the main peak, demonstrating the stability-indicating power of the method. The performance of the method was validated according to the present ICH guidelines for specificity, limit of detection, limit of quantification, linearity, accuracy, precision, ruggedness and robustness.

KEYWORDS: Plerixafor, RP-HPLC, Method Development, Validation, ICH Guidelines.

INTRODUCTION

Plerixafor (PLX) is a bicyclam derivative which is a potent and selective antagonist of the CXCR4 receptor, competing with the latter's cognate ligand SDF-1α (also known as CXCL12). PLX mobilizes human HSC with long-term repopulating ability in immune deficient mice, and acts synergistically with G-CSF for the mobilization of HSC in both mice and humans. Two multicenter, phase III, double-blinded, placebo-controlled studies of PLX have been carried out in patients with lymphoma and myeloma. Two further recent studies of first-line PLX + G-CSF are consistent with these data. PLX +G-CSF are, therefore, a more effective HSC-mobilizing schedule than G-CSF alone. The combination allows the collection of greater numbers of stem cells in fewer apheresis sessions than G-CSF alone, and can salvage those patients who fail to mobilize adequate HSC with chemotherapy-based mobilization or with G-CSF alone. Data available for PLX added to chemotherapy + G-CSF mobilization.[1]

PLX gives better CD34+ cell yields and is less toxic than conventional chemotherapy, and that there is no difference in subsequent clinical outcome. The data make a strong case for preferring PLX + G-CSF to chemotherapy G-CSF for first-line mobilization of lymphoma and myeloma patients requiring stem cell transplantation.

PLX has orphan drug status in the USA and European Union; it was approved by the US Food and Drug Administration in December 2008. PLX is chemically 1-{[4-(1, 4, 8, 11-tetra azacyclotetradecan-1-ylmethyl)} phenyl] methyl}-1, 4, 8, 11-tetraazacyclotetradecane.

Monograph for PLX is not available in European Pharmacopoeia (EP), Japan Pharmacopoeia (JP) and United States Pharmacopoeia (USP) and also no method has been reported for the determination of PLX.[2]

The objective of this article is to present an Analytical RP-HPLC method development and validation for assay of Plerixafor injection. In the present work, a simple, fast and precise liquid chromatographic method was developed and validated for the assay of Plerixafor Injection.[3]

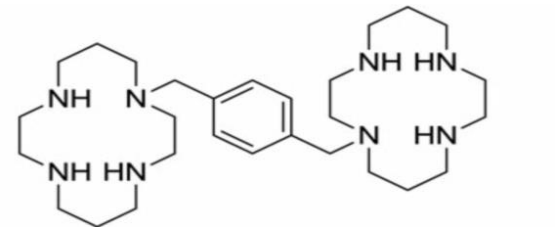

Fig-1: Chemical Structure of Plerixafor.

MATERIALS AND METHODS

Chemicals and reagents

Acetonitrile (HPLC grade), Anhydrous ethanol, Sodium per chlorate monohydrate,1-Octane Sulphonic acid sodium salt anhydrous, HPLC grade water, Perchloric acid were purchased from Merck.

Chromatographic Conditions and Equipment

The LC system of Dionex ultimate 3000 HPLC with a photodiode array detector was used for this study and chromatographic separation was achieved on the Agilent Zorbax eclipse plus C18 column (100mm, 4.6 mm, 3 µm) column as the stationary phase using isocratic method. Mobile phase contains buffer (adjusted to a pH of 2.0 with Perchloric acid) and acetonitrile in the ratio of 65:35% (v/v). The peaks were monitored at the wavelength of 210 nm, keeping the flow rate of 1.0 mL/min the column temperature at 35ºC using injection volume of 10 µL.[4]

EXPERIMENTAL

Preparation of Diluent, Standard and Sample Solution

A mixture of 0.01 N HCl and ethanol in the ratio of 95:5 (v/v) was used as diluent.

Standard stock solutions of PLX were prepared by dissolving appropriate amount standard materials in the diluent and used.

Test sample solution for the assay was prepared at 20 mg/mL, respectively. Test sample solution can be prepared at 1mg/mL and further diluted to 0.1 mg/mL solution for the assay test solution.[5]

Method Development

The main target of the chromatographic method is to develop a new method and the main component PLX with each other. A blended solution containing 20 mg/mL of PLX and 0.1 mg/mL of each standard was prepared in diluent used for the method 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.[21-25]

RESULTS AND DISCUSSION

RESULTS

Method Development

A variety of mobile phases using different columns (Grace Vydac C-18 and Agilent Zorabax Eclipse plus C-18) were investigated in the development of RP-HPLC method suitable for analysis of Plerixafor. These included 0.1%formic acid/ACN as mobile phase, 1-Pentane Sulphonic acid sodium salt and ACN (65:35% v/v) as mobile phase, 1-Octane Sulphonic acid sodium salt and Acetonitrile (65:35% v/v). The suitability of the mobile phase was decided on the basis of binding effect to the stationary phase, the sensitivity of the assay, suitability for stability studies, time required for the analysis, ease of preparation, and use of readily available cost-effective solvents.[6] Trail chromatograms are shown in figure 2 to 6.

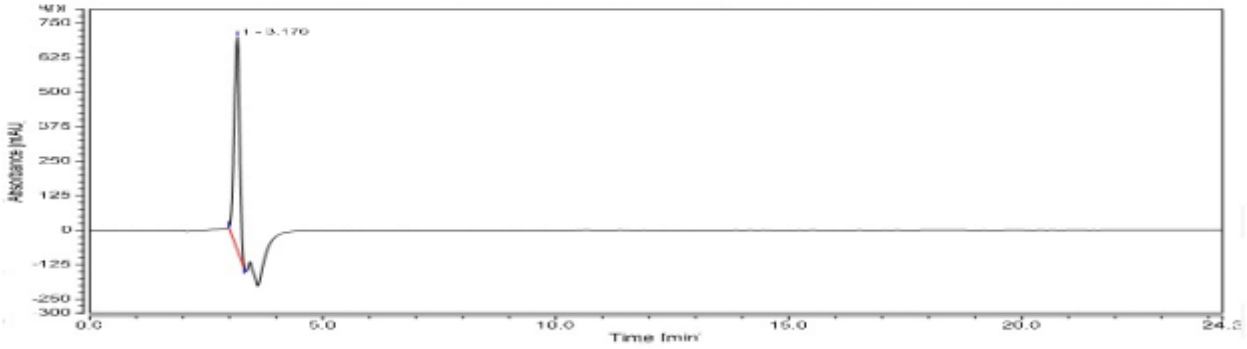

Fig-2: Trial – 1 Chromatogram.

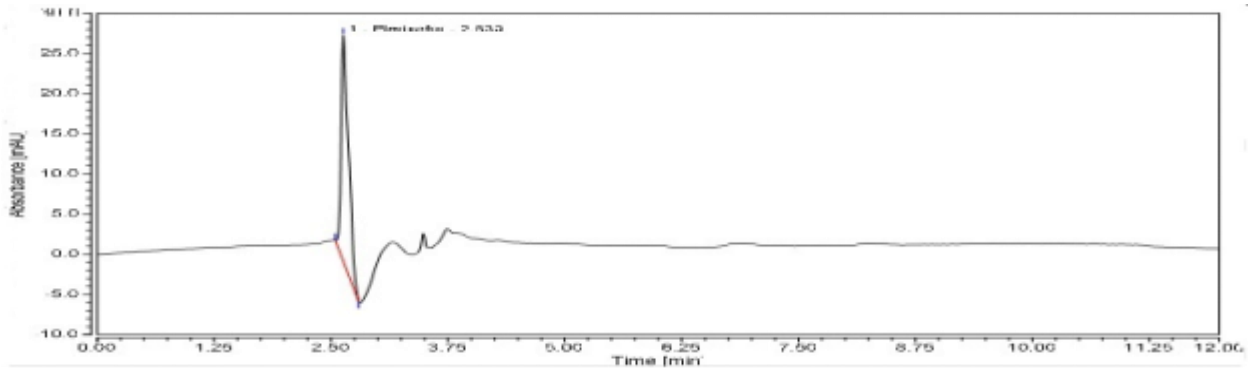

Fig-3: Trial – 2 Chromatogram.

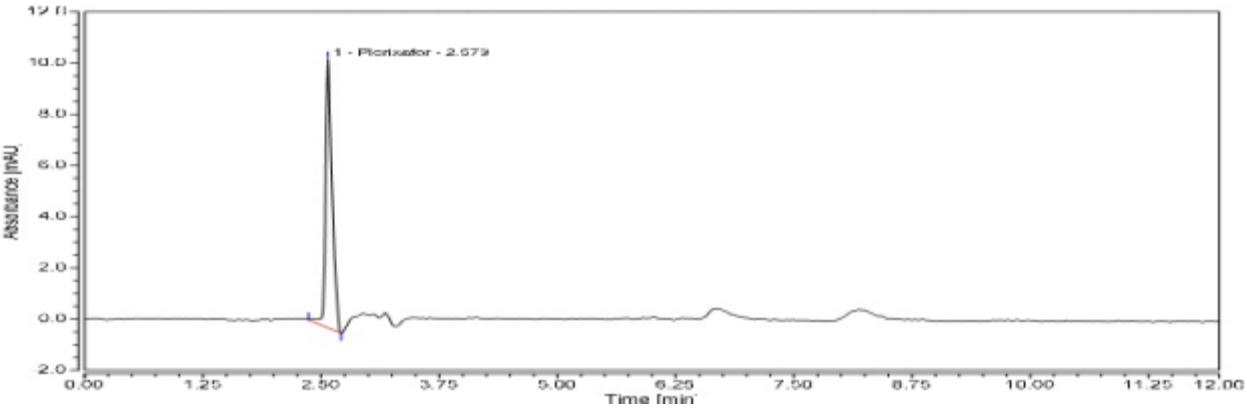

Fig-4: Trial – 3 Chromatogram.

A) A. Method development trail chromatograms using Grace Vydac C-18 column (a. Formic acid buffer mobile phase, b. 1-pentane Sulphonic acid sodium salt buffer mobile phase; and c.1-octane sulfonic acid sodium salt buffer) with pH 2.0.

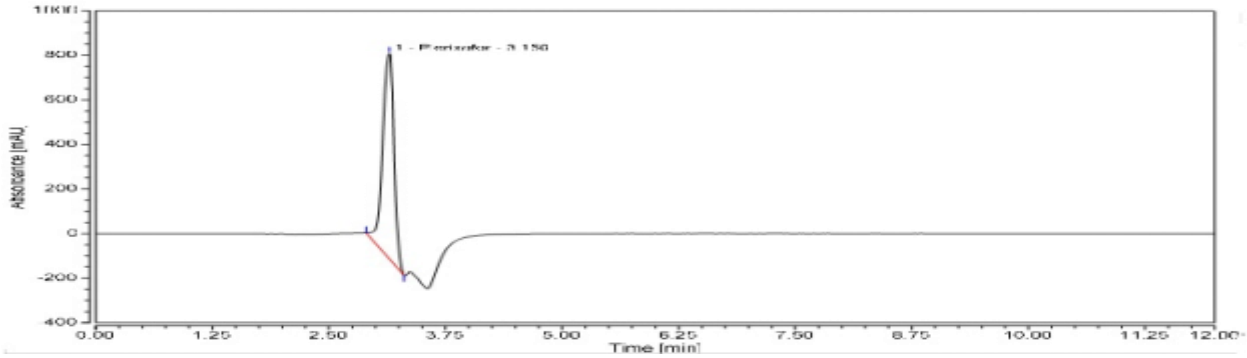

Fig-5: Trial – 4 Chromatogram.

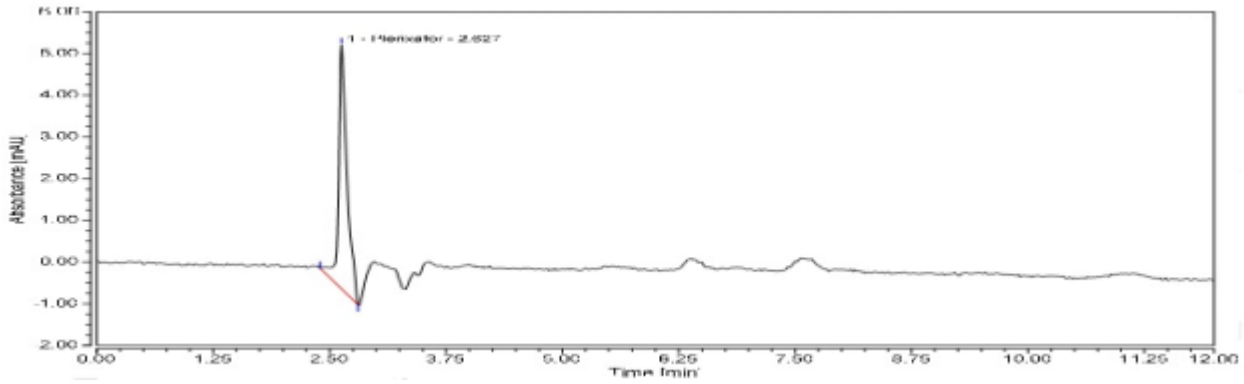

Fig-6: Trial – 5 Chromatogram.

Optimization of Analytical Method

Optimized Chromatographic Conditions

Mobile phase ratio : 1-Octane Sulphonic acid sodium salt (pH-2) & Acetonitrile (65:35% v/v)
Column : Agilent Zorbax Eclipse Plus C_{18} column (100mmx4.6 mm, 3 µm)
Column temperature : 35 ºC
Mode of Elution : Isocratic
Type of Detector : Photodiode Array Detector
Wavelength : 210 nm
Flow rate : 1.0 ml/min
Injection volume : 10 µl
Run time : 12.0 minutes

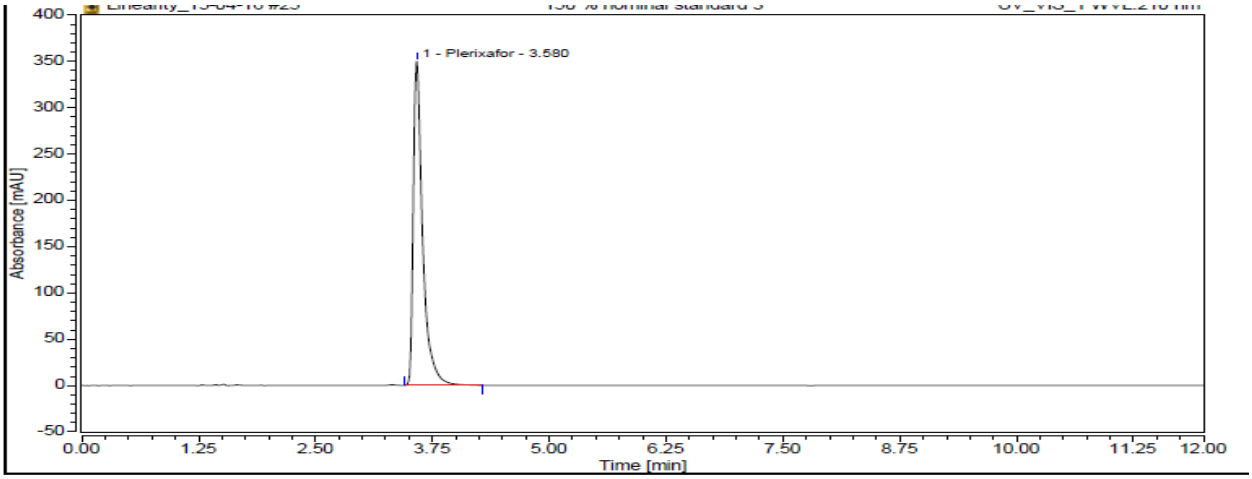

Fig-7: Optimized Chromatographic Condition of Plerixafor.

Method Validation

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of all its potential impurities. To prove the stability-indicating nature of the method, PLX samples were exposed to acid, base, peroxide, heat, water, humidity and photolytic stress conditions, and then injected into the HPLC which is attached to a PDA detector. Peak purity of PLX peak was verified by using the PDA detector in the stress samples and found passing with respect to the empower algorithm. Specificity experiment samples were stressed various stress conditions and analyzed along with unstressed samples.[7-9] The results are shown in tables 1-3.

Table-1: Acid Hydrolysis.

RUN	Peak area	% Recovery	Asymmetry	Theoretical plates
0.01M HCl treated at 50C_24hrs	26.44	96.11	1.65	7562
0.01M HCl treated at 50C_24hrs Spiked	49.40	186.84	1.84	7094
RUN	**Peak area**	**% Recovery**	**Asymmetry**	**Theoretical plates**
0.1M HCl treated at 50C_24hrs	26.36	95.83	1.88	7298
0.1M HCl treated at 50C_24hrs Spiked	50.04	189.85	1.75	7923

Table-2: Base Hydrolysis.

RUN	Peak area	% Recovery	Asymmetry	Theoretical plates
0.1M NaOH treated at 50C_24hrs	26.46	96.19	1.75	7923
0.1M NaOH treated at 50C_24hrs Spiked	49.79	188.18	1.88	7331

Table-3: Oxidation.

RUN	Peak area	% Recovery	Asymmetry	Theoretical plates
3% H2O2 treated	13.21	48.04	1.67	8323
3% H2O2 Spiked	37.32	282.42	1.89	7689

Precision

The precision of the method was verified by repeatability and by intermediate precision. Six replicate sample solutions were prepared for the estimation of assay, the percent assay for each replicate, the average for six replicates and its % RSD were calculated.[10-12] The results were showed in Table 4 and 5.

Table-4: Repeatability Results of Plerixafor.

Repeatability	Plerixafor		
Run	**Area**	**Asymmetry**	**Theoretical Plates**
Injection 1	27.4833	1.79	8038
Injection 2	27.5111	1.82	8022
Injection 3	27.5172	1.79	8045
Injection 4	27.518	1.82	8031
Injection 5	27.5543	1.77	8040
Injection 6	27.5361	1.8	8027
STDEV	**0.02**		
AVG	**27.52**		
%RSD	**0.09**		

Table-5: Intermediate Precision Results of Plerixafor.

Intermediate Precision	Plerixafor		
Run	**Area**	**Asymmetry**	**Theoretical Plates**
Injection 1	27.5612	1.78	7788
Injection 2	27.3824	1.77	7769
Injection 3	27.3498	1.8	7789
Injection 4	27.384	1.76	7773
Injection 5	27.5296	1.78	7780
Injection 6	27.423	1.79	7762
STDEV	**0.09**		
AVG	**27.44**		
%RSD	**0.32**		

Limits of Detection (LOD) and Quantification (LOQ)

The LOD and LOQ for assay were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations.[13]

Detection limit may be expressed as

$$DL = \frac{3.3\sigma}{S}$$

$$QL = \frac{10\sigma}{S}$$

Where σ=the standard deviation of the response

S=the slope of calibration curve

Linearity

Linearity test solutions for the assay method were prepared from the PLX stock solution at six concentration levels from 50 to 150% of the assay analyte concentration. The peak area versus concentration data was treated by least-squares regression analysis. The linearity calibration plot for the assay method was obtained over the calibration ranges tested, and the correlation coefficient obtained was 0.9999.[14-16] The results are shown in Table 7.

Table-6: Results of LOD & LOQ.

LOD	0.05
LOQ	0.15

Table-7: Linearity Readings of Plerixafor.

% Concentration	Mean Area Response
50	13.81
75	20.52
100	27.5
125	33.9
150	40.59
Coefficient Correlation	0.9999
Intercept	0.1743
Slope	0.2705

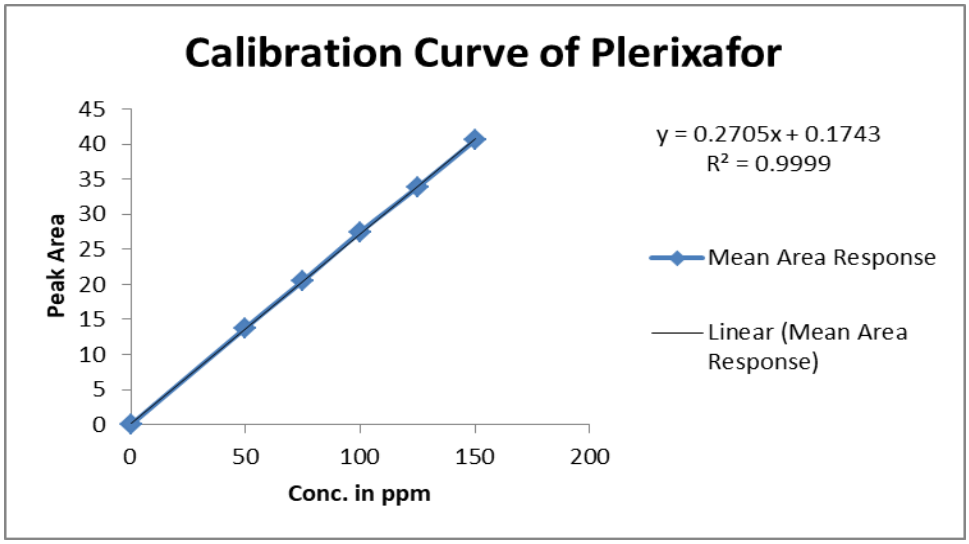

Fig-8: Linearity Graph of Plerixafor.

Accuracy

The accuracy of the assay was evaluated in triplicate at five different concentration levels (50% to 150%) by using drug substance.[17-18] The recovery of PLX from bulk drug ranged from 95-105. The results are shown in Table 8.

Table-8: Accuracy Readings of Plerixafor.

Name of the Injection	Plerixafor		
	Sample Area	Standard Area	%Recovery
80% spiked-1	21.01	21.56	**97.42**
80% spiked-2	21.06	21.56	**97.66**
80% spiked-3	21.01	21.56	**97.43**
100 % spiked-1	26.49	27.21	**97.34**
100 % spiked-2	26.53	27.21	**97.50**
100 % spiked-3	26.57	27.21	**97.62**
120 % spiked-1	32.05	31.64	**101.30**
120 % spiked-1	32.05	31.64	**101.31**
120 % spiked-1	32.07	31.64	**101.36**

Robustness

To determine the robustness of the method, experimental conditions were deliberately changed and the tailing factors and resolution for PLX were recorded. The flow rate of the mobile phase was changed by varying 0.1 units and verified at 0.9 and 1.1 mL/min. The effect of the column temperature on the resolution was studied at 33 and 37ºC instead of at 35ºC. The effect of the percent organic strength was studied by varying acetonitrile ±2%. The effect of pH of mobile phase buffer was studied by varying pH +0.1 units of method pH (2.0) keeping other mobile phase components constant.[19-20] The results are shown in Table No. 9 to 20.

Flow rate

Table 9: Robustness Readings of Plerixafor (0.75 mL).

RUN	Retention time	Peak area	% Recovery	Asymmetry	Theoretical plates
100%Nominal Std	3.64	26.5619	100.32	1.78	7868
Injection 1	3.64	27.476	103.44	1.81	7827
Injection 2	3.64	27.4799	103.46	1.81	7815

Table-10: Robustness Readings of Plerixafor (0.73 mL).

RUN	Retention time	Peak area	% Recovery	Asymmetry	Theoretical plates
100%NominalStd	3.747	27.2959	100.40	1.82	7946
Injection 1	3.747	27.8282	101.95	1.81	7901
Injection 2	3.747	27.73	101.59	1.81	7926

Table-11: Robustness Readings of Plerixafor (0.77 mL).

RUN	Retention time	Peak area	% Recovery	Asymmetry	Theoretical plates
100%NominalStd	3.553	26.4273	102.41	1.76	7774
Injection 1	3.553	26.0791	98.68	1.73	7751
Injection 2	3.55	26.0639	98.62	1.74	7740

Temperature

Table-12: Robustness Readings of Plerixafor (35ºC)

RUN	Retention time	Peak area	% Recovery	Asymmetry	Theoretical plates
100%Nominal Std	3.64	26.5619	100.32	1.78	7868
Injection 1	3.64	27.476	103.44	1.81	7827
Injection 2	3.64	27.4799	103.46	1.81	7815

Table-13: Robustness Readings of Plerixafor (33ºC).

RUN	Retention time	Peak area	% Recovery	Asymmetry	Theoretical plates
100%NominalStd	3.747	26.4799	100.29	1.81	7639
Injection 1	3.747	26.6822	100.76	1.83	7625
Injection 2	3.75	26.675	100.74	1.78	7633

Table-14: Robustness Readings of Plerixafor (37ºC).

RUN	Retention time	Peak area	% Recovery	Asymmetry	Theoretical plates
100%NominalStd	3.54	26.5857	100.82	1.79	7952
Injection 1	3.54	26.593	100.03	1.74	7922
Injection 2	3.54	26.4174	99.37	1.74	7952

Organic Modifier

Table-15: Robustness Readings of Plerixafor (35% ACN).

RUN	Retention time	Peak area	% Recovery	Asymmetry	Theoretical plates
100%NominalStd	3.64	26.5619	100.32	1.78	7868
Injection 1	3.64	27.476	103.44	1.81	7827
Injection 2	3.64	27.4799	103.46	1.81	7815

Table-16: Robustness Readings of Plerixafor (33% ACN).

RUN	Retention time	Peak area	% Recovery	Asymmetry	Theoretical plates
100%NominalStd	3.773	26.4842	99.70	1.76	7558
Injection 1	3.773	26.4591	99.91	1.77	7538
Injection 2	3.773	26.4125	99.73	1.76	7510

Table-17: Robustness Readings of Plerixafor (37% ACN)

RUN	Retention time	Peak area	% Recovery	Asymmetry	Theoretical plates
100%Nominal Std	3.42	26.548	100.45	1.76	7492
Injection 1	3.42	26.5088	99.85	1.83	7452
Injection 2	3.43	26.5312	99.94	1.8	7465

pH

Table-18: Robustness Readings of Plerixafor (pH-2)

RUN	Retention time	Peak area	% Recovery	Asymmetry	Theoretical plates
100%NominalStd	3.64	26.5619	100.32	1.78	7868
Injection 1	3.64	27.476	103.44	1.81	7827
Injection 2	3.64	27.4799	103.46	1.81	7815

Table-19: Robustness Readings of Plerixafor (pH-1.9).

RUN	Retention time	Peak area	% Recovery	Asymmetry	Theoretical plates
100%NominalStd	4.303	26.606	99.32	1.79	7923
Injection 1	4.307	26.4603	99.45	1.76	7918
Injection 2	4.31	26.5045	99.62	1.79	7914

Table-20: Robustness Readings of Plerixafor (pH-2.1)

RUN	Retention time	Peak area	% Recovery	Asymmetry	Theoretical plates
100%NominalStd	4.297	26.5723	100.72	1.75	7609
Injection 1	4.313	26.3644	99.22	1.78	7613
Injection 2	4.33	26.4168	99.41	1.78	7607

Solution Stability

The solution stability of PLX in the assay method was carried out by leaving both of the test solutions of the sample and standard solutions in tightly capped volumetric flasks at room temperature for 24 h. The same sample solutions were tested up to 24 hrs. The % RSD values for the PLX assay during the solution stability and mobile phase stability experiments were within 1. No significant changes in the amounts of the test solutions of the sample and standard solutions during the solution stability (on the bench top).[26] The results are shown in Table-21.

Table-21: Solution Stability Studies of Plerixafor.

DAY	STD results (2-8 C)	% Difference	Sample result (2-8 C)	% Difference	STD Result (RT)	% Difference	Sample result(RT)	% Difference
DAY 0	99.51	**0.00**	98.89	**0.00**	_	_	_	_
DAY 1	99.60	**0.09**	98.45	**-0.44**	99.66	**0.78**	98.59	**-0.30**
DAY 2	99.59	**0.08**	98.71	**-0.18**	98.99	**0.10**	98.59	**-0.31**
DAY 3	99.65	**0.14**	98.83	**-0.06**	99.06	**0.17**	98.70	**-0.19**
DAY 4	99.58	**0.07**	98.78	**-0.11**	99.30	**0.41**	99.19	**0.30**
DAY 5	101.17	**1.67**	100.27	**1.39**	100.74	**1.87**	100.76	**1.89**
DAY 6	100.45	**0.94**	100.02	**1.14**	100.09	**1.21**	100.07	**1.19**
DAY 7	100.66	**1.16**	100.10	**1.22**	100.46	**1.59**	99.68	**0.80**
DAY 15	103.78	**4.29**	103.29	**4.45**	104.44	**5.61**	103.36	**4.52**

DISCUSSION

Method Development and Optimization

The main target of the chromatographic method is to achieve develop new analytical method and the main component PLX with each other. Wavelength was selected based on wavelength maxima of PLX UV spectrums. PLX have wavelength maxima between 200 to 220 nm. Hence the 210 nm was selected as suitable wavelength for the assay of PLX.

A blended solution containing 20 mg/mL of PLX and 0.1mg/mL of each standard was prepared in diluent which was used for the method's development.

Initial experiments were performed with 0.1% of formic acid buffer and 95% acetonitrile as mobile phase, using Agilent Zorbax eclipse plus(C-18, 100-mm, 4.6-mm, 3.5µ particles) column and found no retention of PLX. PLX was not retained even with the mobile phase with composition of 1-pentane sulfonic acid sodium salt and acetonitrile (65:35%) as mobile phase. PLX is not retaining on column by using normal buffers may be because of the high polarity of compounds. Hence further experiments were tried using the ion-pair reagents in the mobile phase.[27]

Ion-pairing agents are ionic compounds that contain a hydrocarbon chain that imparts a certain hydrophobicity interactions between analyte and stationary phase; therefore, analyte can be retained on a reversed-phase column. Ion-pair reagents also help in changing the selectivity, improving analyte solubility and suppressing unwanted interactions.

Ion-pair reagents are available in acidic (anionic) and basic (Cationic) forms. Quaternary ammonium salts or alkyl amines are called as acidic or anionic ionic pair reagents and useful in separation of acidic samples.

Alkyl sulfonates are called as basic or cationic ion-pair reagents and useful in separation of basic samples.

PLX is basic in nature, hence the use of cationic ion-pair reagent is recommended over anionic ion-pair because basic samples can be better separated using a straight-chain alkyl sulfonic acid in the mobile phase. The ion-pair reagent 8mM of 1-Octane sulfonic acid sodium salt of cationic nature along with 45mM of sodium per chlorate (adjusted to a pH of 2.0 with diluted Perchloric acid) was used as mobile phase and found capable of retaining PLX in the column for more than 10 min.[28]

Method Validation

Forced Degradation Studies

The stress conditions used for the degradation study of PLX includes acid hydrolysis (1 N HCl at 50ºC for 24 h and at 25ºC for 24 h), basic hydrolysis (1 N NaOH at 50ºC for 24 h and at 25ºC for 24 h), and oxidation (3% H2O2 at 50ºC for 24 h and at 25ºC for 24 h).

Precision

The repeatability of the test method for the assay was checked by a six replicate injection of standard solution (0.1 mg/mL). The RSD (%) of peak area was calculated. Inter- and intra-day variation and analyst variation were studied to determine the intermediate precision of the proposed method. With these acceptable results the proposed method is precise.

Limit of Detection and of Quantification

LOD is defined as the lowest amount of analyte that can be detected, but not necessarily quantitated as an exact value, whereas limit of quantification is defined as the lowest amount of analyte that can be determined with suitable precision and accuracy. Both LOD and LOQ values are determined using the signal-to noise ratio method.

Linearity

The linearity of an analytical procedure is its ability to obtain test results that are directly proportional to the concentration of the analyte in the sample. The linearity of the test method was established from the LOQ to 150% of the test concentration for PLX. The result showed that an excellent correlation existed between the peak area and concentration of the analyte. This confirmed the linear relationship between peak areas and concentrations. The results indicate very good linearity.[29]

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the reference value and the value found. Assay was determined in triplicate from the LOQ to 0.1% of the PLX test concentration. All results are within an acceptable limit. We can say the method is accurate.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. In all the varied chromatographic conditions (flow rate, column temperature, organic composition and pH of the mobile phase), the results are within acceptable limits.[30]

Solution Stability

The results from the solution stability experiments confirmed that the standard solutions and test solutions in the diluents were stable for up to 24 h during the determination of assay.

CONCLUSION

The present study successfully developed and validated a rapid, reliable, and stability-indicating RP-HPLC method for the quantitative analysis of PLX drug substance. The method demonstrated excellent system suitability and was found to be highly precise, accurate, linear, robust, and specific in accordance with regulatory guidelines. The optimized chromatographic conditions provided well-resolved peaks with a retention time of less than 12 minutes, ensuring efficient analysis.

Furthermore, the method effectively distinguished the drug from its degradation products, confirming its stability-indicating capability. Owing to its simplicity, reproducibility, and short analysis time, the proposed method is suitable for routine quality control analysis of PLX in bulk as well as for stability studies during storage and manufacturing processes.

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