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Research Article

Quantification Of Analytical Method For The Estimation Of Selexipag And Its Impurities By Rp-Hplc

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ABSTRACT

A simple, rapid, precise RP-HPLC method has been developed and validated for the quantification of the impurities of Selexipag. Selexipag is used for the treatment of pulmonary arterial hypertension (PAH) to delay disease progression and reduce risk of hospitalization. Chromatography was carried out using Waters C18 250 mm x 4.6mm, 5µ column with a flow rate of 1.0 mL/min. The elution was carried out using mobile phases 0.1% OPA buffer and acetonitrile. The developed method has been validated for the various parameters like Precision, Accuracy, Linearity, Robustness, LoD, LoQ, Solution stability. The stability-indicating capability of the method was established by forced degradation studies under stress conditions like acid, base, peroxide, UV, thermal, humidity. The retention times of Impurity 2, Selexipag, Impurity 1, and Impurity 3 were found to be 2.378, 3.380, 4.918, and 5.630 respectively. The method was specific and linear $R^2 > 0.999$. The LoD and LoQ were found to be 0.38µg/mL and 0.116µg/mL for SEL, 0.068µg/mL and 0.206µg/ml for IMP 1, 0.034µg/mL and 0.102µg/mL for IMP 2, and 0.094µg/mL and 0.852µg/mL for IMP 3 respectively. The mean % recovery obtained was found to be 99.31% for SEL, 99.41% for IMP 1, 99.31 % for IMP 2 and 99.62 % for IMP 3 respectively. The chromatographic method developed for the estimation was said to be rapid, simple, specific, sensitive, precise, accurate, robust, and reliable that can be effectively applied for routine analysis in research institutions, quality control departments in industries for the identification and estimation of the impurities of Selexipag.

Keywords: Method development, Validation, Selexipag, RP-HPLC, Impurities

INTRODUCTION :

Selexipag is an antihypertensive used in the treatment of pulmonary arterial hypertension (PAH) which in-turn interrupt the progression of disease resulting in the reduction of risk of hospitalization. PAH is a relatively rare disease with usually a poor prediction requiring more treatment options to prolong long-term outcomes. [1] The active metabolite of SelexipagACT-333679 (MRE-269) along with the drug act as agonists of the prostacyclin receptor which causes increase of vasodilation in the pulmonary circulation resulting in the reducing of elevated pressure in the blood vessels. [2] USFDA approved Selexipag on December 22, 2015. [3] Selexipag is metabolized to ACT-333679 an active metabolite which is 37 times more potent than Selexipag with the help of carboxylesterase- 1. Selexipag is chemically distinct and with high selectivity for the IP receptor. Selexipag and its metabolite are selective for the IP receptor over the other prostanoid receptors. Chemical structures of Selexipag, and its impurities 1, 2, 3 given in fig 1, 2, 3 & 4 respectively.
The above mentioned are the chemical structures of the three impurities of selexipag [4-6]. Extensive literature review was conducted to identify the various methods proposed for the development and validation of selexipag and its impurities [7-9]. It was identified that only few analytical HPLC [10-11], UV [12], and LCMS [13] methods have been reported for the estimation of selexipag individually and or along with drug combinations in

pharmaceutical preparations. Therefore, a successful attempt has been made to develop RP-HPLC method which would be able to separate and quantify a combination of selexipag with its three impurities in a single run and validated as per the ICH guidelines Q3A(R2), Q2(R1) [14-15].

MATERIALS AND METHODS:

Equipment:
Waters HPLC 2695 separation module equipped with Waters 2998 photodiode array detector. The data acquired was processed using Windows Empower-2 software. The materials were weighed using Denver electronic balance, pH measurements were done by using digital pH meter 7007. Ultrasonic bath, Vacuum pump, Hot air oven were used. For the spectroscopic data UV-VIS spectrophotometer integrated with UV Win 6 Software (PG Instruments T60) was used.

Chemicals:
HPLC grade solvents include acetonitrile, water and methanol were purchased from E. Merck chemical division, Mumbai, India. Analytical grade chemicals include Potassium dihydrogen ortho phosphate, Tri Ethyl Amine, Ortho-phosphoric acid and Sodium dihydrogen ortho phosphate were purchased from Rankem, avantor performance material India limited.

Chromatographic conditions:
HPLC analysis was carried out on Waters Alliance-HPLC system equipped with 2695-separation module and the data was acquired by Empower® version 2. Successful elution was accomplished by using Waters C18 250 mm x 4.6mm, 5 µm as a column with mobile phase 0.1% OPA buffer and acetonitrile in gradient mode. Injection volume was 10 µL, flow rate was maintained at 1.0mL/min with runtime of 7 min and the temperature was maintained at 30 °C throughout the analysis. Detection and purity establishment of the drugs were achieved using PDA detector at 260 nm wavelength. Optimized chromatographic conditions were listed in Table 1. Gradient programming was given in Table 1.1.

Preparation of Selexipag standard solution:
0.5 mg of Selexipag drug is weighed accurately and transferred to a 10 mL clean dry volumetric flask, 5 mL of diluent was added and sonicated for 10 minutes to dissolve. The final volume was made up with the diluent and filtered through 0.45 µ nylon filter to obtain the solution with a concentration of 50 ppm. From the above stock solution 0.2 mL was pipetted out in to a 10 mL volumetric flask and then made up to the final volume with diluent to obtain a concentration of 1ppm solution.

Preparation of impurities standard solutions:
0.5 mg of Impurities working standards A, B, and C were accurately weighed and transferred to a 10 mL clean dry volumetric flask, 5 mL of diluent was added and sonicated for 10 minutes to dissolve. The final volume was made up with the diluent and filtered through 0.45 µ nylon filter to obtain a concentration of 50 ppm solutions.

Preparation of mobile phase:
1000 mL of Acetonitrile is considered as mobile phase A and 1000 mL of Phosphate buffer solution of pH 3.0 was considered as mobile phase B. The solutions were sonicated for 30 min to degas. Gradient mode of elution was carried out using both mobile phases A and B in various proportions. Table 1.1 summarizes the gradient program used for the elution.

Preparation of diluent:
Phosphate buffer (pH 3.0) and Acetonitrile were mixed in the ratio of 50:50 and sonicated for 30 min to gas and used as diluent.

Preparation of blank:
0.1% OPA buffer, Acetonitrile were taken in the ratio 70:30. The solution was injected into HPLC as blank and the chromatogram was recorded.

Method Validation:
RP-HPLC method which was optimized was now validated according to ICH guidelines Q2(R1) in order to determine the system suitability, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, ruggedness and robustness.

System suitability:
System suitability parameters were evaluated to determine the performance of the system. 10 µL of standard solution was injected five times, and the chromatograms were recorded.

Specificity:
The specificity of the analytical method was determined by injecting the solutions of blank, placebo, working standards of selexipag and impurities individually to investigate interference from the representative peaks.

Precision:
Repeatability/method precision was performed by injecting six replicates of same concentrations of selexipag and impurities, % assay and %RSD were calculated for each compound.
Reproducibility/Ruggedness was determined using the same concentrations of solutions with different analysts and on a different instrument on the different day in the same laboratory.

Accuracy:
Accuracy was evaluated using spiking method. The recovery studies were carried out by adding known amounts (50%, 100% and 150%) of the working standard solutions of selexipag and impurities to the pre-analysed sample. In order to determine the accuracy, the solutions were prepared in triplicates and injected into the system.

Linearity:
Linearity was evaluated by analyzing different concentrations of the standard solutions of selexipag and impurities. Six working standard solutions ranging between 0.25 - 1.50 µg/mL for Selexipag and its three impurities were prepared and injected. The response found to be a linear function of concentration over peak area and subjected to regression analysis to calculate the calibration equation and correlation coefficient.

Limit of detection and limit of quantification:
Limit of detection (LoD) and limit of quantification (LoQ) of selexipag and impurities were determined by calibration curve method. Solutions of selexipag and impurities were prepared in linearity range and injected (n = 3).

Robustness:
The robustness of the developed method was determined by deliberately changing the experimental conditions. Parameters like resolution, tailing factor, and theoretical plates of selexipag and impurities peaks were evaluated. To study the outcome of the flow rate on the developed method, it was changed ± 0.1 mL/minute. The effect of column temperature was studied at difference of ± 5 °C.

Solution Stability:
10 µL of standard solution was injected six times to determine the solution stability at 0 hrs (initial) and at 24 hrs (final). The chromatograms were recorded and the system suitability parameters were evaluated.

Forced Degradation Studies:
To evaluate the stability-indicating property of the developed method stress studies were performed by considering selexipag and impurities working standard solutions of concentrations 1µg/mL each. Intended degradation was attempted by exposing the solutions for the stress conditions like photolytic stress (1.2 million lux hours followed by 200 Watt hours), heat (exposed at 105 °C for 6 hours), acid (2 N Hcl for 2 hours at 60 °C), base (2 N NaoH for 2 hours at 60 °C), oxidation (20% peroxide for 30 minutes at 60 °C), water (refluxed for 12 hours at 60 °C), and humidity (exposed to 85% RH for 72 hours).

RESULTS AND DISCUSSION:

System Suitability:
The retention times of Impurity 2, Selexipag, Impurity 1, and Impurity 3 were found to be 2.378, 3.380, 4.918, and 5.630 min respectively and the respective chromatogram was showed in figure 5. The column efficiency for selexipag and its impurities peaks was identified from the theoretical plate count which is more than 3000, tailing factor less than 2.0, and the %RSD was found to be less than 2.0%. The resolution of the peaks of selexipag and impurities were also found to be in the limits (>1.5). The results were summarized in table 2. Chromatogram of mixture of selexipag with impurities represented in fig 5.

Specificity:
From the obtained chromatograms it can be inferred that there were no co-eluting peaks at the retention time of selexipag and impurities which shows that peak of analyte was pure and the excipients in the formulation did not interfere with the analyte of interest.

Precision:
From the results summarized in table 3, it is evident that % assay for selexipag and impurities was found to be in the range of 98 – 102 %, and the % RSD for selexipag and impurities to be within 2 %. Hence the method is precise, reproducible and rugged for 48 hours' study. The results of precision were summarized in Table 3.

Accuracy:

It is identified from the results summarized in table 4, that the % recovery for selexipag and impurities found to be in the range of 98 –102 % and the % RSD less than 2 %. Hence the proposed method was accurate. The results of accuracy were summarized in Table 4.

Linearity:

Linearity was evaluated by analysing different concentrations of standards. From the results tabulated in table 5, it is inferred that the correlation coefficient was greater than 0.999. The slope and y-intercept values also confirmed good linearity between peak areas and concentration. The standard curves of selexipag and impurities were shown in fig 6 to 9.

LoD and LoQ:

The Limit of Detection and Limit of Quantification of selexipag and impurities were calculated by using following equations (ICH, Q2 (R1)) and the LoD and LoQ values are reported in table 6. These LOD = 3.3 × σ/S and LOQ = 10 × σ/S. Where σ = the standard deviation of the response and S = slope of the calibration curve. The results of LoD and LoQ were summarized in Table 6.

Robustness:

From the results it is inferred that the system suitability parameters such as resolution, %RSD, tailing factor, and the theoretical plate count of selexipag and impurities remained unaffected by deliberate changes. Thus, the method was found to be robust with respect to variability in applied conditions.

Solution stability:

Results indicates that the system suitability parameters at 0 hrs (initial) and at 24 hrs (final) are within the acceptable limits according to ICH guidelines which indicates that the standard solution was stable till 24 hrs. The results were summarized in tables 7 and 8.

Forced Degradation Studies:

The results which were shown in table 9 indicates that the degradation was not observed in photolytic stress, humidity, acid, base, water hydrolysis, and thermal stress studies. The peak purity of selexipag and its impurities was found to be homogeneous based on the evaluation parameters such as purity angle and purity threshold. It is also concluded that there is no interference from degradants, which was determined from the peak purity and thus facilitates error-free quantification of selexipag and the impurities. Hence, the method is considered to be “stability-indicating.”

CONCLUSION:

A simple and robust RP-HPLC method has been developed for the simultaneous estimation of Selexipag and its three impurities. The proposed method was validated in accordance with ICH guidelines considering all the parameters which include system suitability, specificity, precision, linearity, LOD, LOQ, accuracy and robustness. The method was found to be specific to separate the peaks of selexipag and its three impurities with better resolution. Thus, the obtained data prove the effectiveness of the proposed RP-HPLC method for the separation of three impurities with the selexipag, which can be adopted in routine analysis in pharmaceutical industries.

CONFLICT OF INTEREST:

The authors declare no conflicts of interest.

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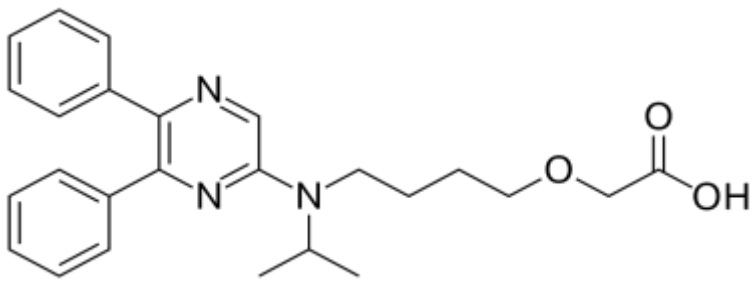

Figure 1. Chemical structure of selexipag

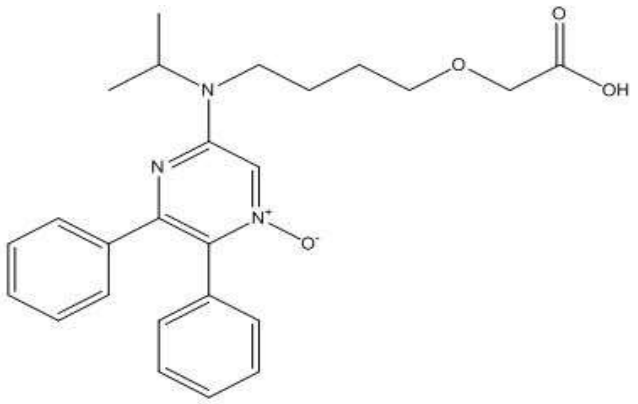

Figure 2. Chemical structure of selexipag impurity 1

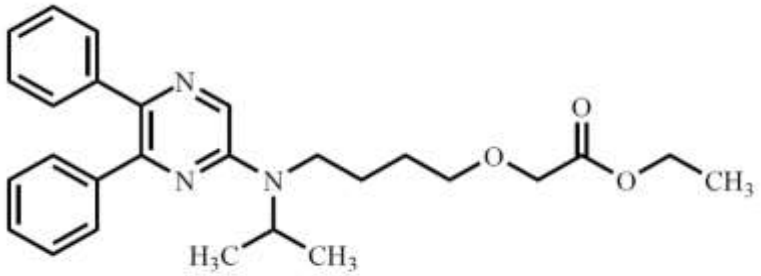

Figure 3. Chemical structure of selexipag impurity 2

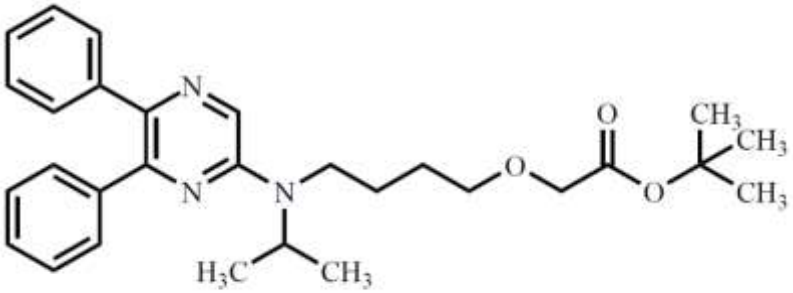

Figure 4. Chemical structure of selexipag impurity 3

Table 1: Optimized Chromatographic Conditions:

Parameter	**Optimized conditions**
Stationary phase (column)	Waters C18(250 mm x 4.6mm, 5µ)
Mobile phase	Acetonitrile and Phosphate buffer (pH 3.0) in gradient mode.
Flow rate	1 mL/min
Injection volume	10 µL
Column temperature	30°C
Detection	Performed at 260 nm using PDA detector
Run time	10 min
Diluent	Acetonitrile : pH 3.0 Phosphate buffer (50:50)

Table 1.1: Gradient programming

S. No	**Time(min)**	***A (%)**	***B (%)**
1	0.01	70.0	30.0
2	1.0	70.0	30.0
3	2.0	60	40
4	3.0	55	45
5	5.0	50	50
6	7.0	40	60
7	9.0	70	30
8	10.0	70	30

*A- Acetonitrile, *B – pH 3.0 Phosphate buffer

Table 2: System suitability data

Parameter	Impurity 2	Selexipag	Impurity 1	Impurity 3	Acceptance criteria
RT (min)	2.378	3.380	4.918	5.630	--
USP Plate count*	7465	8628	1758	14302	NLT 3000
%RSD	1.0	0.8	0.8	1.1	NMT 2.0
Peak Tailing*	1.40	1.2	1.27	1.26	NMT 2.0
Resolution*	--	7.6	9.2	3.8	>1.5

* = Average of 6 replicate injections

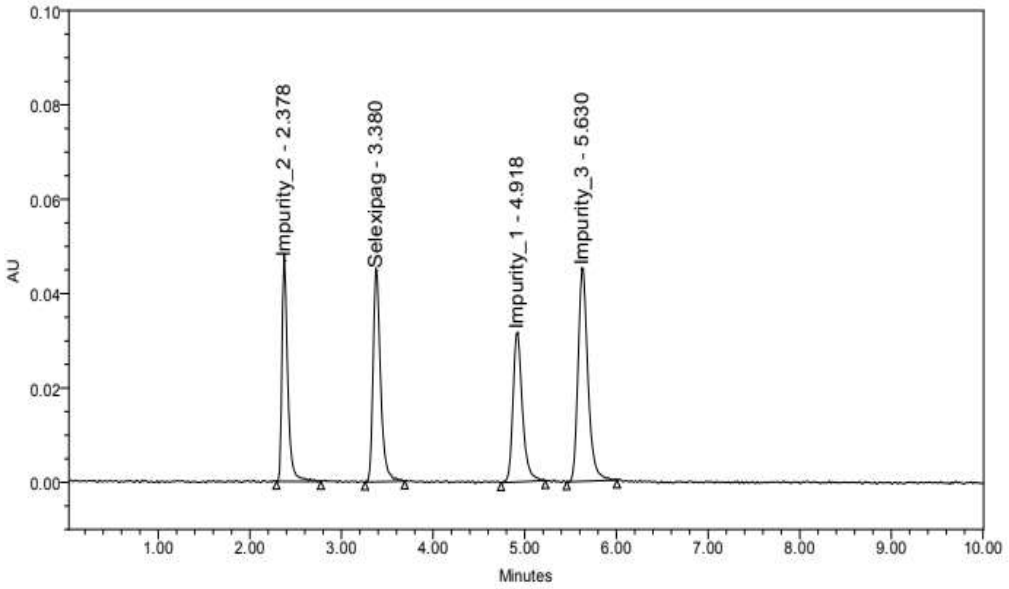

Figure 5. Chromatogram of Selexipag with impurities

Table 3: Method Precision data

S.NO	Peak Areas	% Assay	Peak Areas	% Assay	Peak Areas	% Assay	Peak Areas	% Assay
	IMPURITY-2		SELEXIPAG		IMPURITY-1		IMPURITY-3	
1	209712	98.81	265235	99.62	236408	100.25	346161	100.58
2	213141	100.42	266766	100.2	236204	100.16	342701	99.59
3	213133	100.51	268114	100.58	233159	98.87	345905	100.51
4	210303	99.09	266560	100.12	239168	101.42	342668	99.58
5	211429	99.62	262534	98.61	235712	99.95	346732	100.75
6	215663	101.61	268141	100.71	234205	99.31	340701	98.99
Mean	212230	100.15	266225	99.97	235809	100.72	344144	100.61
SD	2197	1.03503	2109	0.77034	2069	0.87738	2449	0.71155
% RSD	1.04	1.03	265235	99.62	0.88	0.86	0.71	0.72

Table 4: Accuracy data

Drug name	Conc. (%)	Amount spiked (µg/mL)	Amount recovered (µg/mL)	% recovery	Statistical parameters
Selexipag	50 100 150	0.05 0.10 0.15	0.048 0.095 0.142	99.59 99.31 99.03	Mean % recovery: 99.31 %RSD: 0.7
Impurity- 2	50 100 150	0.05 0.10 0.15	0.048 0.095 0.142	99.59 99.31 99.03	Mean % recovery: 99.31 %RSD: 0.7
Impurity- 1	50 100 150	0.05 0.10 0.15	0.049 0.098	100.17 99.70 98.38	Mean % recovery: 99.41 %RSD: 0.7

			0.145		
Impurity- 3	50 100 150	0.05 0.10 0.15	0.048 0.095 0.142	100.30 100.08 98.49	Mean % recovery: 99.62 %RSD: 0.8

Table 5: Linearity data of 3 impurities

S.No	Impurity 2		Selexipag		Impurity 1		Impurity 3	
	Conc (µg/mL)	Peak area*	Conc (µg/mL)	Peak area*	Conc (µg/mL)	Peak area*	Conc (µg/mL)	Peak area*
1	0.25	59120	0.25	88843	0.25	69659	0.25	96471
2	0.5	109195	0.5	140802	0.5	122195	0.5	175128
3	0.75	161600	0.75	207259	0.75	178835	0.75	255452
4	1.0	211930	1.0	267013	1.0	234970	1.0	345270
5	1.25	254011	1.25	329149	1.25	284833	1.25	414165
6	1.50	306005	1.50	386383	1.50	332889	1.50	504686
7	Regression equation y = 196480x + 11723 R² = 0.9992		Regression equation y = 241428x + 25325 R² = 0.9993		Regression equation y = 212594x + 17877 R² = 0.9991		Regression equation y = 325486x + 13728 R² = 0.9992	

* = Average peak area of 3 replicate injections for each concentration

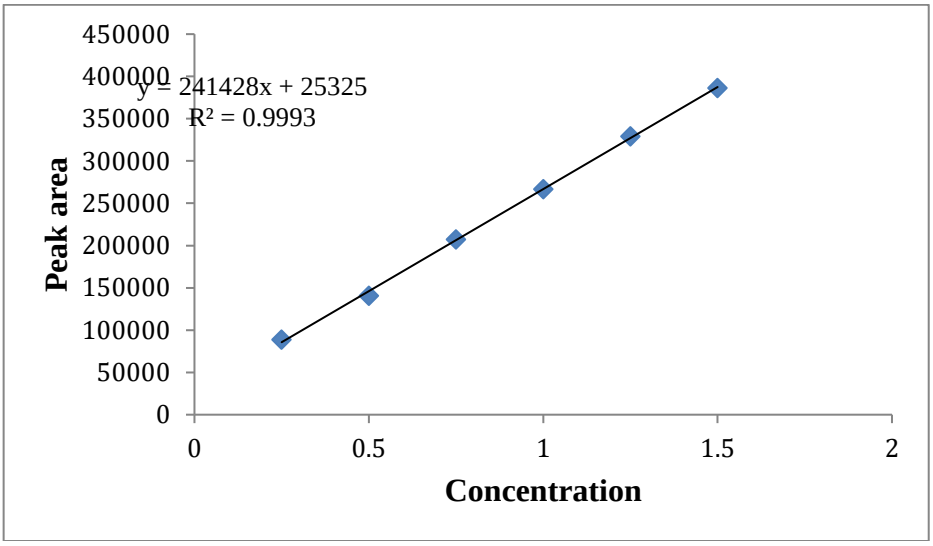

Fig. 6. Standard curve of Selexipag

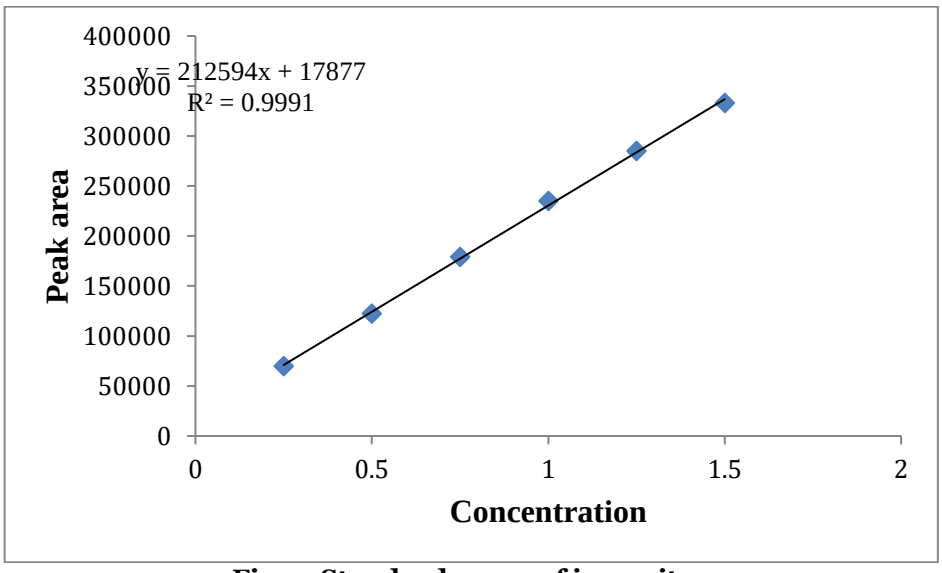

Fig. 7. Standard curve of impurity 1

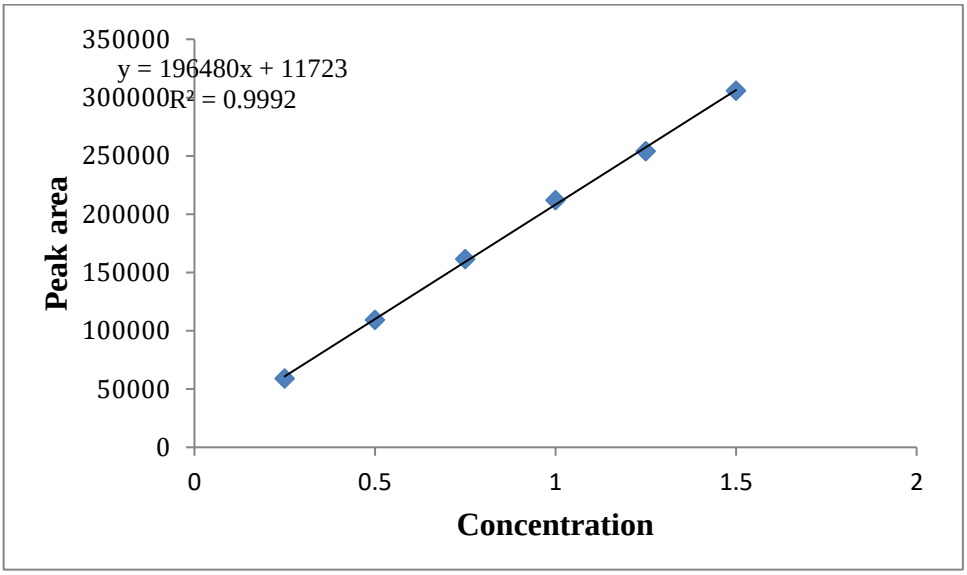

Fig. 8. Standard curve of impurity 2

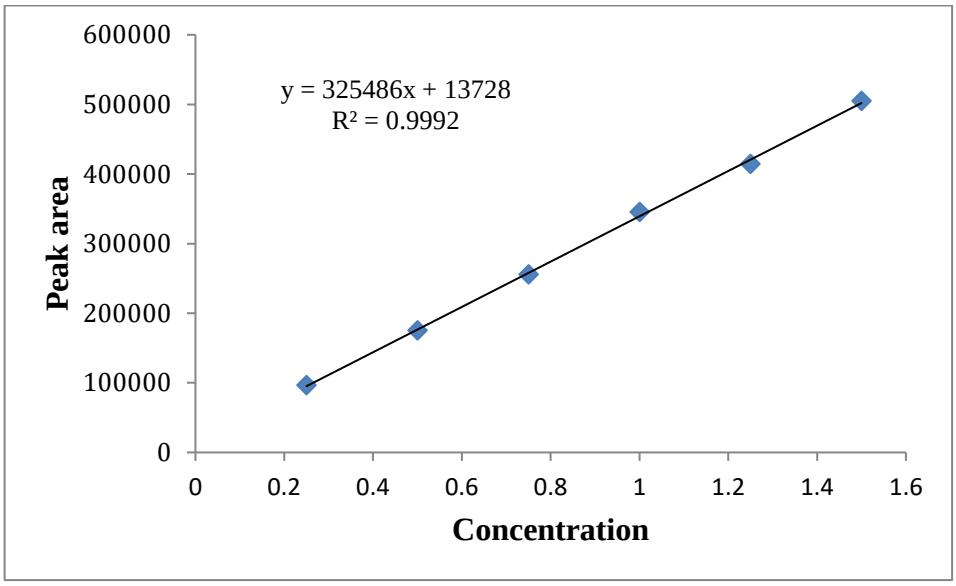

Fig. 9. Standard curve of impurity 3

Table 6: LoD and LoQ data

Drug name	LoD (µg/mL)	LoQ (µg/mL)
Selexipag	0.38	0.116
Impurity 1	0.068	0.206
Impurity 2	0.034	0.102
Impurity 3	0.094	0.285

Table 7: System suitability data for solution stability at 0 hrs

S.NO	Peak name	RT*	Peak area*	Plate count*	Peak tailing*	Resolution *
1	Impurity-2	2.378	202426	7824	1.4	-
2	Selexipag	3.380	253984	8731	1.3	7.5
3	Impurity-1	4.918	225226	11490	1.2	9.1
4	Impurity-3	5.630	332644	14250	1.3	3.8

* = Average of 6 replicate injections

Table 8: System suitability data for solution stability at 24 hrs

S.NO	Peak name	RT*	Peak area*	Plate count*	Peak tailing*	Resolution *
1	Impurity-2	2.491	175309	6884	1.35	--
2	Selexipag	3.486	243607	8596	1.33	7.1

3	Impurity-1	5.059	212987	12303	1.26	9.1
4	Impurity-3	5.865	313651	14340	1.25	4.2

* = Average of 6 replicate injections

Table 9. Forced degradation studies at different stress conditions

Stress Condition	% Degradation	Purity Angle	Purity Threshold
Selexipag			
Acid	5.53	1.930	2.390
Base	4.02	2.002	2.478
Peroxide	3.30	2.366	2.708
Thermal	2.64	1.900	2.343
Photo Stability	1.65	2.207	2.760
Water	0.82	2.624	3.009
Impurity 2			
Acid	5.28	1.120	1.380
Base	4.61	1.080	1.449
Peroxide	3.82	13.334	1.732
Thermal	2.47	1.088	1.351
Photo Stability	1.74	1.175	1.485
Water	0.79	1.189	1.411
Impurity 1			
Acid	5.79	0.749	1.025
Base	4.48	1.213	1.513
Peroxide	3.87	1.920	2.356
Thermal	2.53	1.144	1.470
Photo Stability	1.65	1.383	1.808
Water	0.80	1.507	1.936
Impurity 3			
Acid	5.90	1.303	1.728
Base	4.42	1.216	1.615
Peroxide	4.23	1.273	1.616
Thermal	3.06	1.263	1.587
Photo Stability	1.55	1.567	1.979
Water	0.86	1.311	1.678

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