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Stability indicating LC-MS/MS method and validation of Selexipag impurities and identification of its force degradation products

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ABSTRACT

Selexipag belongs to a class of medicines known as IP prostacyclin receptor agonists used to treat pulmonary arterial hypertension. A simple and sensitive LC-MS/MS method has been developed for the identification of process related impurities in Selexipag API. The method was developed using a Zorbax C18 15 × 0.46 cm, 5μ column with a gradient program at 35 ^{0}C and flow rate of 1.0 mL/min. Detection was carried out by MS/MS with an ESI detector. Stress conditions were established by exposing the drug to acidic, alkaline, oxidative, thermal and photolytic stress condition. Identified and unidentified impurity was found when the fractions of acid and alkaline degradation product was analyzed by LC-MS/MS. The suggested methodology can be used to test the quality of Selexipag and identify the process-related impurities in pharmaceutical products.

Introduction

Pulmonary arterial hypertension (PHA) is a major public health problem in the developed countries recently. It is a life-threatening condition that gets worse over time and can eventually lead to heart failure [1–2]. Selexipag is used to treat pulmonary arterial hypertension, a condition that affects the heart and lungs. Selexipag is orally selective prostacyclin receptor agonist causing vasodilation in the circulatory system and reducing pressure build-up in blood cells that transport blood from the heart to the lungs. Selexipag has been recently (2015) approved in several countries for the long-term treatment of PHA in adult patients. The drug significantly reduces the risk of death, hospitalization, long-term oxygen therapy, and lung transplantation [3]. General side effects of Selexipag such as headache, jaw pain, and hyperthyroidism have been reported. However, Selexipag can be used to treat PHA due to its high selectivity for IP receptors and fewer side effects compared with other PGI_2 receptor antagonists. According to the literature review, several analytical techniques are available to detect of Selexipag and its related substances in bulk and dosage form and also in rate plasma using HPLC and LC-MS [4–10]. Similar to this, a few spectroscopic methods have been published for the quantification of Selexipag with MBTH via oxidative coupling in pharmaceutical formulations [11–16]. However, there are no suitable method available for the analysis of Selexipag and its related impurities in single or in combined drug products using LC-MS and HPLC methods. In this context, study is focused on the developing an effective stability indicating LC-MS/MS approach for quantification and validation of Selexipag as well as the characterization of its degradation products. The chemical structure of Selexipag and its related impurities shown in Fig. 1.

Experimental

Materials and reagents

Selexipag API (purity 99.3%), Selexipag impurity A (purity 99.7%), Selexipag Methyl ester (purity 98.8%) & Selexipag acid (purity 97.7%) was acquired as a gift from Shilpa Medicare. All the chemicals and reagents used for method development were LC-MS grade and acquired from J.T. Baker, Mumbai, India. Buffer solutions were made with LC-MS grade water from Aquarch.

Instrumentation

The chromatographic measurements were carried out on a Shimadzu LC system with binary pump (LC-20AT), degasser unit (DGU-20A5), and SIL-HTC auto sampler (Shimadzu Corporation, Japan). Quantification was done by mass detection using an AB Sciex mass spectrometer (Model: API-2000 – Foster City, USA) equipped with an ESI interface at

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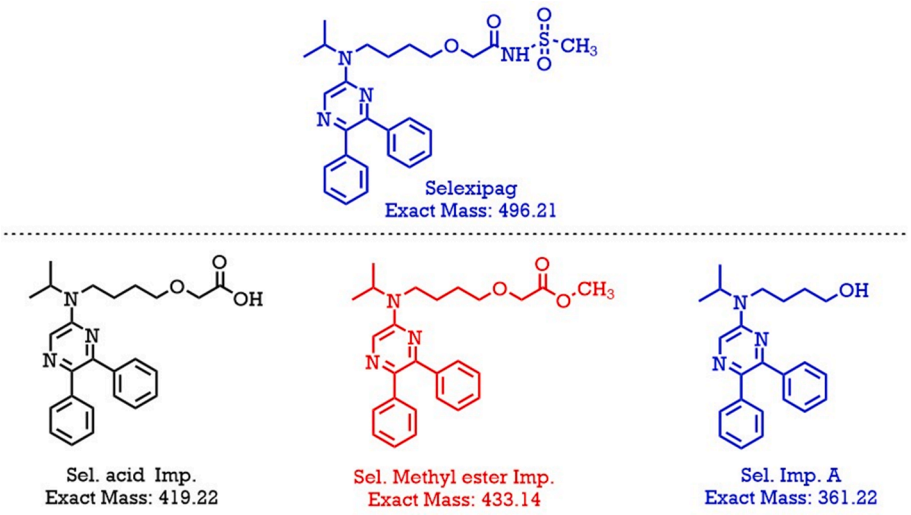

Fig. 1. The chemical structure of Selexipag and its related impurities.

400 °C. A positive ion detection mode with a 5000 V ion spray voltage was set for detection. The source parameters were as follows: nebulizer gas (GS1), 50 psi; drying gas (GS2), 30 psi. The compound parameters: delustering potential, 15 V; entrance potential, 10 V; collision energy, 15 V; collision cell exit potential, 10 V. The detection of analytes was carried out in the multiple-reaction monitoring (MRM) mode. Quadrupoles Q1 and Q3 were set to unit resolution. pH of the buffer solutions was tested with a Labtronics LT-10 digital pH meter. The pH of all solutions was tested using an Ultrabasic potentiometer (Denver, CO, USA). Deaeration was carried out using Thornton T50 ultrasonic bath (Meltser-Toledo, Bedford, MA).

Chromatographic conditions

Waters LC system was used for method development and validation. The separation was carried out using a Zorbax C18 15 × 0.46 cm, 5µ analytical column maintained at ambient condition. The mobile phase comprised of two parts: Mobile phase-A (0.1 % formic acid) and mobile phase-B (acetonitrile) with an initial ratio of 50:50 (% v/v). The mobile phase was degassed in an ultra-sonication bath and filtered through a 0.22 µm nylon membrane filter before use. A 1.0 mL/min flow rate of mobile phase was used. The injection volume was set at 20 µL. Step gradient program was used to conduct the analysis. Detection was performed by MS/MS with ESI electron spray ionizer detector. Analyst software™ (Version 1.6.2) was used for data acquisition and processing.

Sample preparations

Preparation of standard stock, and sample solutions

Diluent (used for preparation of standard and sample solution) was prepared by mixing buffer (0.1% formic acid) and Acetonitrile in a 70: 30 (v/v) ratio.

Preparation of standard stock solutions

a. **Selexipag standard stock solution (100 µg/mL):** Transfer 10.0 mg of Selexipag to a 100 mL volumetric flask. Add 60 mL of diluent, sonicate to dissolve, and adjust the volume with diluent.
b. **Selexipag standard solution (1.0 µg/mL):** Take 1 mL of Selexipag standard stock solution into 100 mL volumetric flask, adjust the volume with diluent and mix well.

Preparation of impurity standard stock solutions

a. **Selexipag acid standard stock solution (100 µg/mL):** Transfer 5.0 mg of Selexipag acid to 50 mL volumetric flask. Add 30 mL diluent, sonicate to dissolve, then dilute to the desired volume.
b. **Selexipag methyl ester standard stock solution (100 µg/mL):** Transfer 5.0 mg of Selexipag methyl ester to a 50 mL volumetric flask. Add 30 mL diluent, sonicate to dissolve, then dilute to the desired volume.
c. **Selexipag impurity-A standard stock solution (100 µg/mL):** Transfer 5.0 mg of Selexipag impurity-A to a 50 mL volumetric flask. Add 30 mL diluent, sonicate to dissolve, then dilute to the desired volume.
d. **Preparation of impurity standard solution of mixtures of Selexipag acid (1.0 µg/mL), Selexipag methyl ester (1.0 µg/mL) and Selexipag impurity-A (1.0 µg/mL):** Take 1 mL of Selexipag acid, Selexipag methyl ester and Selexipag impurity-A stock solution, transfer to 100 mL volumetric flask and adjust the volume with diluent and mix well.

Preparation of sample solution

Transfer about 10.0 mg of Selexipag API to a 10 mL volumetric flask. Add 5 mL of diluent, sonicate it, and adjust the volume with diluent.

Methodology for the evaluation (Validation)

The analytical performance parameters such as specificity, linearity, range, accuracy, precision, sensitivity (LOQ and LOD), and robustness were validated according to ICH Q2B and FDA guidelines [17–20].

Specificity

Specificity is necessary study to measure the analyte in the presence of its potential impurities. In this study, the specificity was evaluated by spiking diluent, standard and test samples with known impurities. Furthermore, forced degradation study was performed on SEL (1000 µg/mL) sample solution to verify the specificity of the method. The forced degradation study was performed as follows: Acid and basic hydrolysis tests was carried out in 1.0 M HCl and 1.0 M NaOH at room temperature for 4 h, respectively. The oxidative stress study was performed at room temperature for 4 h with 10 mg of solid SEL sample dissolved in 3% H_2O_2 solution. Photolytic degradation study was performed by exposing the 10 mg of SEL to UV light (254 nm) for 4 h. Thermal stress study was carried by placing 10 mg of SEL sample in a hot air oven at 100 ^{0}C for 4 h.

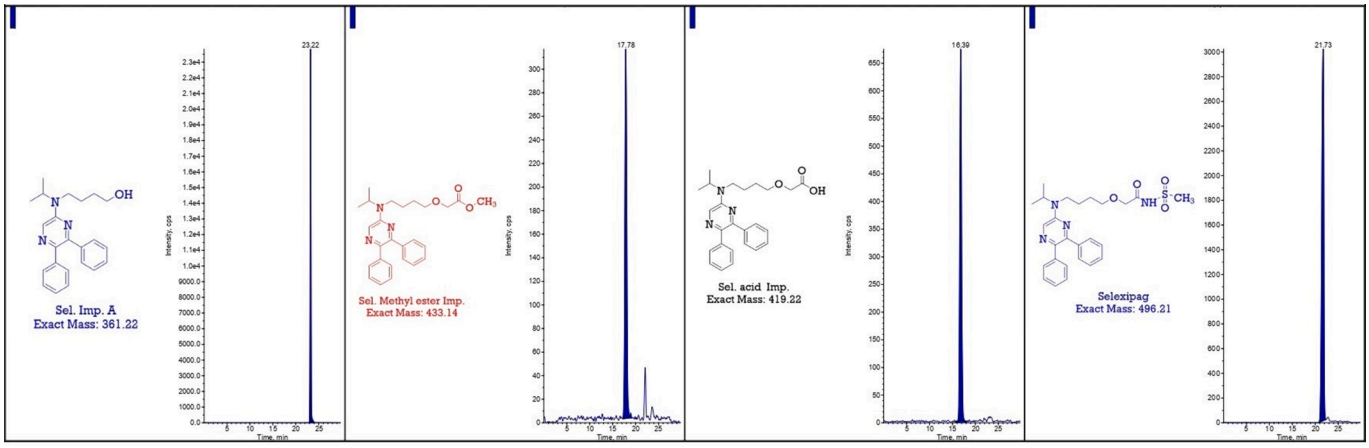

Fig. 2. Final optimized LC chromatogram of Selexipag and its related impurities.

Sensitivity (LOD and LOQ)

The limit of detection (LOD) and limit of quantitation (LOQ) of the impurities were determined from signal to noise ratio values by injecting a series of diluted sample solutions with known concentrations of each impurity. LOD is lowest detection of concentration with 3:1 S/N ratio approximately, while LOQ is lowest quantification of concentration with 10:1 S/N ratio approximately.

Linearity

To evaluate the linearity of the method, calibration curve for each impurity were constructed by injecting six different test sample solutions of impurities in triplicate. Six different test sample solutions of impurities were prepared from the standard stock solutions to achieve different concentration levels (LOQ, 50%, 75%, 100%, 125% and 150%). For each analyte, correlation coefficients (r^2) were calculated using a calibration curve. The slop of the calibration curves was used to calculate the relative response factor (RRF).

Accuracy

The accuracy of Selexipag and its related impurities was carried out by analyzing four different concentration levels testing solutions of each impurity (LOQ, 80%, 100%, and 120%) in triplicate (n = 3) with the time interval of 24 h.

Precision

The precision of the method was studied by determination of method (repeatability) precision and intermediate precision (ruggedness). For injection repeatability, six replicate (n = 6) standard preparation at 100 % level were injected individually on the same day whereas intermediate precision was checked by injecting six replicate standard preparation with the time interval of 24 h. The peak area of analytes was measured and % RSD was calculated at the acceptance threshold < 2.0%.

Robustness

In order to evaluate the robustness of the method, the chromatographic conditions were deliberately changed to check the system suitability parameters and % difference in impurity values. The robustness of the method was determined through the study of the effect of small changes in the flow rate of the mobile phase (±0.2 mL/min) and organic composition (±2%).

Result and discussion

Method development and optimization

In order to achieve the selectivity of the analytical method, the selection of a suitable mobile phase was done by performing a sequential trial of the different mobile phases on selected Agilent Zorbax C18 15 × 0.46 cm, 5μ column. At the initial stage, 0.1% formic acid (mobile phase-A), and methanol (mobile phase-B) in a ratio of 50:50 (v/v) was selected as mobile phase and a trial was run on C18 column at the flow rate of 1.0 mL/min under the isocratic mode. The result reveal that no chromatographic peaks were eluted from the C18 column. Further trial was conducted using 0.1% formic acid in water (mobile phase-A), and acetonitrile (mobile phase-B) in the ratio of 50:50 (v/v) using C18 column. The result showed that SEL, SEL acid imp., and SEL imp. A peaks were eluted from the column with retention times of 32.83, 26.47 and 24.24 min, respectively but SEL methyl ester imp. peak did not elute from the column until 35 min. The further trial was conducted using 0.1% formic acid in water (mobile phase-A), and acetonitrile (mobile phase-B) in the ratio of 40:60 (v/v). The result showed analyte and impurities peaks were eluted within 25 min but SEL acid imp. and SEL imp. A, peaks found co-eluted. Again, the trial was conducted with the ratio of 45:55 (v/v) and result showed that SEL methyl ester imp. peak eluted at around 38.5 min while SEL acid imp. & SEL imp. A, peaks found co-eluted. Hence linear gradient program was tried to achieve a peak resolution. Gradient trial was conducted with initial mobile phase ratio of 50:50 (v/v) and kept it isocratic up to 20 min and then increase % of acetonitrile up to 90% (v/v). The result reveled that all the analyte

Table 1
Gradient program.

Time	Mobile phase-A (%)	Acetonitrile-B (%)
0–20	50	50
20–20.01	10	90
20.01–25	10	90
25–25.01	50	50

Table 2
Forced degradation conditions applied to selexipag.

Conditions	Solvent	Temperature	Standing time	Degradation product
Acid hydrolysis	1.0 M HCl	Room temperature	4 h	SEL acid imp
Alkaline hydrolysis	1.0 M NaOH	Room temperature	4 h	Unknown imp
Oxidation	3% H_2O_2	Room temperature	4 h	None
Thermal	Solid state	100 ^{0}C	4 h	None
Photolysis	Solid state	Room temperature	4 h	None

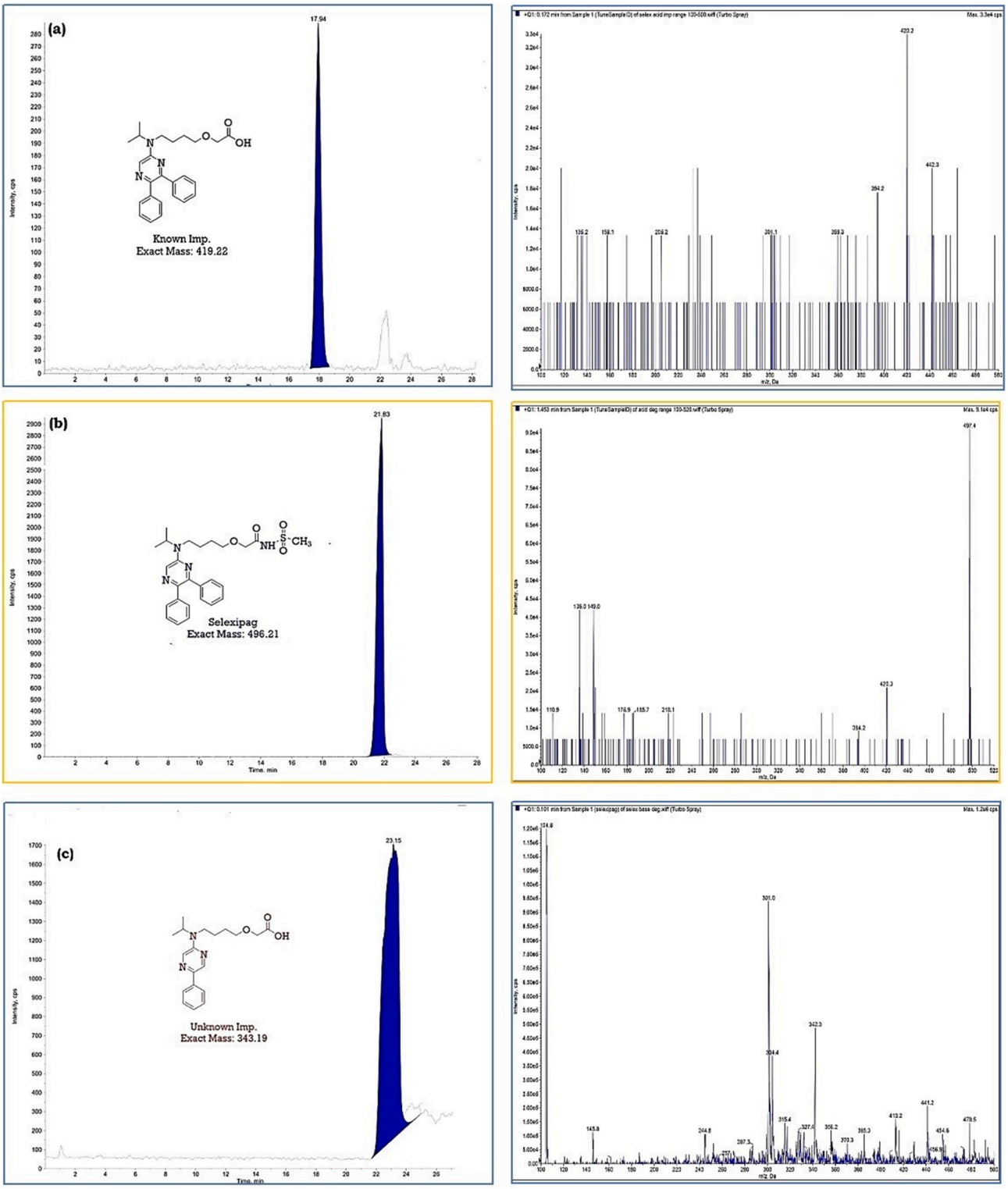

Fig. 3. LC-MSMS graphs of selexipag acid and alkaline fractions.

peaks were well resolved, eluted within 25 min of run time and complies the system suitability. A representative LC chromatogram and optimized gradient program of Selexipag and its related impurities is shown in Fig. 2 and Table 1.

Method validation

Specificity

For the specificity study, selexipag was exposed into thermal, photolytic, alkaline, acid and oxidative degradation conditions (Table 2). Each forced degradation condition was compared to a blank solution. The results reveled that no significant degradation was noted when selexipag was subjected to thermal, photolytic, and oxidative

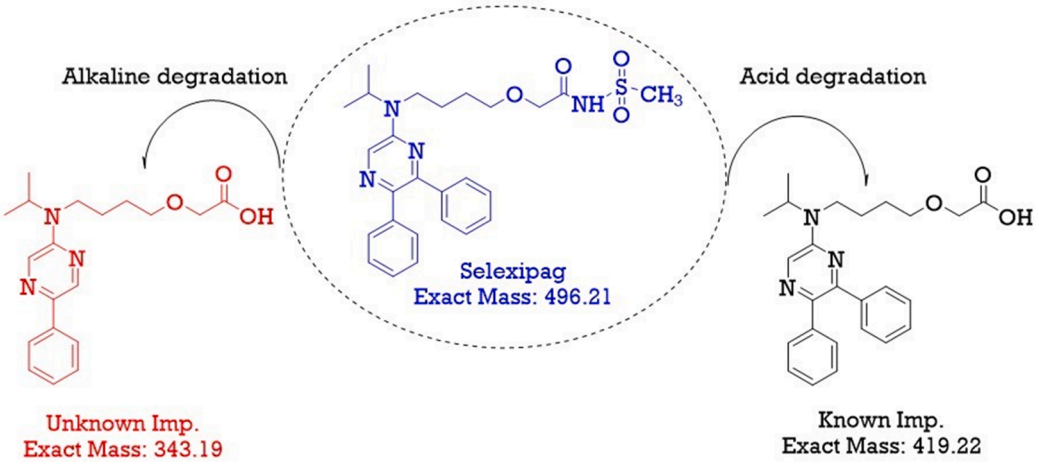

Fig. 4. Degradation pathway of the selexipag.

Table 3
LOD, LOQ, regression analysis of calibration graphs for Selexipag and its related impurities.

Parameters	SEL	SEL imp – A	SEL acid imp.	SEL methyl ester imp.
LOD (μg/mL)	0.12	0.14	0.14	0.06
LOQ (μg/mL)	0.37	0.42	0.42	0.18
Concentration Range (μg/mL)	0.4–1.5	0.4–1.5	0.4–1.5	0.2–1.5
Slope	97,371	19,268	8899.7	257,780
Correlation coefficient (r^2)	0.9936	0.9943	0.9938	0.9973
Relative Retention factor (RRF)	NA	0.20	0.09	2.65

degradation. However, apparent degradation was found under acidic and alkaline condition. One known and unknown impurity was found when the fractions of the acidic and alkaline degradation product was analyzed by LC-MS/MS (Fig. 3). Based on the results, we concluded that known impurity was selexipag acid imp (m/z = 420.20 & exact mass = 419.22) which was formed due to the hydrolysis of sulfonamide group during the acid degradation while unknown impurity (m/z = 342.20 & exact mass = 343.19) formed due to the hydrolysis of sulfonamide as well as pyrazine ring during the alkaline degradation. Based on the above studies, potentially related substances were tracked and the formation pathway were described in Fig. 4. The entire evidence further confirmed that the specificity of the developed method for the intended use.

Sensitivity (LOD and LOQ)

The LOD and LOQ of Selexipag and its related substances were

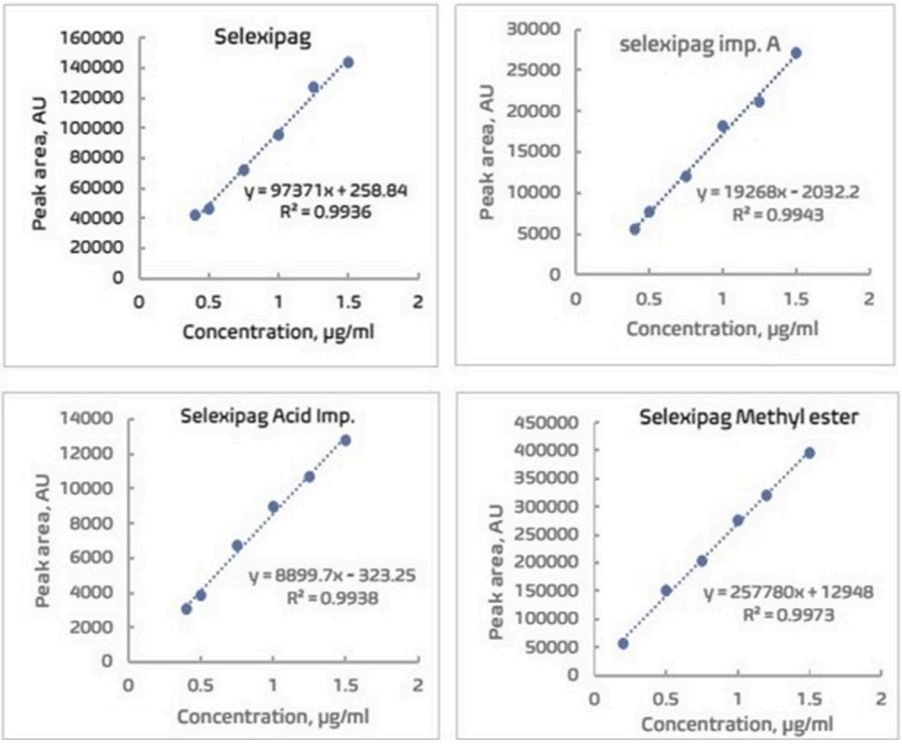

Fig 5. Linearity curves of selexipag and its related impurities.

Table 4
The data of accuracy were obtained by the recovery analysis of Selexipag and its related substances (mean ± SD, n = 3). The data of precision were obtained by the relative standard deviation of peak areas (RSD %, n = 6).

Analytes	Accuracy		Precision		
	Concentration (μg/mL)	Recovery (74–103%, n = 3)	Concentration (μg/mL)	(RSD % ≤ 2, n = 6)	
		24 hrs		Intraday	Interday
SEL acid imp.	0.4 – LOQ	88.7 ± 2.32	1.0	1.87	1.96
	0.8	100.6 ± 1.95			
	1.0	102.2 ± 2.93			
	1.2	101.0 ± 1.22			
SEL imp. A	0.4 – LOQ	74.6 ± 1.375	1.0	1.30	1.63
	0.8	100.6 ± 3.16			
	1.0	102.4 ± 2.70			
	1.2	101.4 ± 2.80			
SEL Methyl ester imp.	0.2 – LOQ	103.3 ± 1.54	1.0	1.07	1.12
	0.8	101.5 ± 0.96			
	1.0	100.7 ± 2.34			
	1.2	101.7 ± 1.23			

Table 5
Comparison variable parameters with method precision.

Parameters	Variation	SEL Imp. A %RS as per the method		SEL Acid Impurity %RS as per the method		SEL Methyl ester %RS as per the method	
		0.10		0.10		0.11	
		%RS	% Diff.	%RS	% Diff.	%RS	% Diff.
Flow rate	(+0.2 mL)	**0.11**	0.01	**0.11**	0.01	**0.11**	0.00
	(−0.2 mL)	**0.12**	0.02	**0.11**	0.01	**0.10**	0.01
Organic solvent	(+2.0 mL)	**0.11**	0.01	**0.11**	0.01	**0.11**	0.00
	(−2.0 mL)	**0.11**	0.01	**0.10**	0.00	**0.11**	0.00
Result		**Complies**		**Complies**		**Complies**	

determined by injecting a series of diluted solutions with known concentrations. The lowest concentration could be reliably detected by the comparison of measured signal with baseline noise signal. Consequently, the LOD and LOQ values of Selexipag were 0.12 and 0.37 μg/mL, respectively. The results demonstrated that this method was sufficiently sensitive, as listed in Table 3.

Linearity

Linearity was described in terms of the calibration curve. The curves of selexipag and its related impurities were obtained by plotting the mean peak areas (mV) against the corresponding concentrations (mg/mL). The linearity of the method was demonstrated by preparing a different concentrated solution from a stock solution. Equation of the calibration curve for selexipag (in the range of 0.4–1.5 μg/mL), SEL acid imp (in the range of 0.4–1.5 μg/mL), SEL imp. A (in the range of 0.4–1.5 μg/mL), and SEL methyl ester imp (in the range of 0.2–1.5 μg/mL) were $y = 97371x + 258.84$, $y = 8899.7x - 323.25$, $y = 19268x - 2032.2$ and $y = 257780x + 12948$, respectively. Each linear regression coefficient was found ≥ 0.99 for calibration curve, displaying excellent correlations between the peak area and concentration (Table 3 and Fig. 5.).

Accuracy

Recovery experiments were performed to evaluate the accuracy of the method by analyzing four different concentration levels (LOQ, 80%, 100%, and 120%) of samples in triplicate with the time interval of 24 h. The percentage recoveries were calculated from calibration curves and recovery rates of Selexipag and its related impurities were at the range of 74 to 103%, more detailed information given in Table 4. These results confirmed that recoveries of the assay method were accurate enough.

Precision

The precision of the developed method was tested by analyzing intra day and inter day studies in six replicates standard samples of Selexipag impurities at 100 % level. The data collected and processed on two consecutive days are presented in Table 4. The %RSD values for intra day and inter day studies were found to be within acceptable limits (<2%). These results showed that the analytical method is precise.

Robustness

The robustness of the method was studied by deliberate small changes in chromatographic parameters such as variation in flow rate (±0.2 mL) and variation in organic solvent (±2.0 %). The results are summarized in Table 5. The result showed that method is robust within acceptable limits.

Conclusion

A simple and sensitive LC-MS/MS approach has been successfully applied for the identification and quantification of selexipag and its related substances, with satisfactory results. Separation of Selexipag and its process related impurities has been carried out using a C18 column with a less retention time. Identified and unidentified impurities were detected under acidic and alkaline stress conditions and analyzed by LC-MS/MS. Validation studies showed that method was specific, accurate and sensitive for the determination of Selexipag and its related substances. As a result, developed method can be successfully used for quality testing of selexipag and determining its process-related impurities in bulk and dosage form.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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