Bioanalytical LC-MS/MS method for Determination and comparison of Selexipag Assay in various Biological materials and its Application to Pharmacokinetics Studies in Rat plasma

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ABSTRACT

A rapid, sensitive and selective bioanalytical method was developed and validated by Liquid Chromatography - Mass spectrometry (LC-MS/MS) for determination and comparison of Selexipag% assay in various biological materials. Selexipag was extracted and compared its % assay after protein precipitation technique from various biological materials such as rat plasma, rabbit plasma, human plasma and urine. Ambrisentan was selected as internal standard. Selected analytical column Waters, X-Bridge C18 3.5 μ (150 x 4.6 mm), mobile phase consists of Hexane sulfonic acid and Acetonitrile (80:20 v/v) at a flow rate of 1.0 mL /min in isocratic mode and Selexipag was determined by the +ve mode of electrospray ionization by using Mass detector. The method was developed to assess the lower limit of detection (LLOD)(0.5 ng/mL), lower limit of quantification(LLOQ) (5 ng/mL) concentrations and Linearity range of 1 ng/mL to 20 ng/mL concentration with regression correlation coefficient 0.999 were observed for Selexipag in Rat plasma. The test samples at lower, medium and higher concentrations of Selexipag shows precision (% CV was 0.8 to 1.11) and accuracy results (97.3% to 100.6%) for inter-day and intra-day analysis at 1, 5, 10, 15 ng/mL concentrations of Selexipag. Appreciable recoveries for Selexipag were observed when extracted in Rat plasma compared with other biological materials. Stability of Selexipag exists in all conditions like wet extract, bench top, freeze-thaw and in instrument auto sampler as per FDA guidelines.

INTRODUCTION

Pulmonary arterial hypertension (PAH) is one the dangerous disease caused by high pulmonary arterial pressure in the presence of a normal or reduced cardiac output (Galie et al., 2009). It has known that, for the treatment of PAH of prostacyclin analogues used due to dysregulation of prostacyclin pathways involved in the pathogenesis of PAH. In fact, the first targeted PAH therapy to be approved was a synthetic prostacyclin analogue, Epoprostenol, which
was executed as a regular intravenous infusion method (Galiè et al., 2003; Barst et al., 1996; Sitbon et al., 2002). However, this is a very complex execution process caused serious side-effects lead to limit its use for the treatment of PAH (Christman et al., 1992). Alternatives to Epoprostenol some other drugs were used in the USA including treprostinil and iloprost, which also have some weakness with respect to injection site pain (treprostinil), frequent dosing (iloprost), and typical prostanoid-related side-effects such as diarrhea, jaw pain, flushing and headache (Barst et al., 2009; Mubarak, 2010). An oral prostacyclin treatment method would be a major favor for PAH treatment. However, most of the oral prostacyclin analogues, such as treprostinil and beraprost, have failed to display a stubborn treatment response as shown by the primary efficacy end-point (Simonneau et al., 2012).
Table 1: Method development Trails

| Sl. No. | Mobile Phase (v/v) | Column | Observation |
|---------|-------------------|--------|-------------|
| Trial-1 | 0.1% OPA: ACN (20:80) | Zorbax C18 (250x4.6mm, 5.0μ) | Peak retention time is very low in Figure 3. |
| Trial-2 | 0.1% OPA:ACN (50:50) | Zorbax C18 (250x4.6mm, 5.0μ) | Base line not sufficient in Figure 4. |
| Trial-3 | 0.1% TEA pH-2.5 with OPA and ACN (20:80) | Waters X-Bridge C18 (150x4.6mm, 3.5μ) | Peaks resolution is very low in Figure 5. |
| Trial-4 | 2.5gm Hexane Sulfonic acid in 1lt water adjusted pH-2.5 with OPA: ACN (50:50) | Waters X-Bridge C18 (150x4.6mm, 3.5μ) | Peaks resolution is very low in Figure 6. |
| Trial-5 | 2.5gm Hexane Sulfonic acid in 1lt water adjusted pH-2.5 with OPA: ACN (80:20) | Waters X-Bridge C18 (150x4.6mm, 3.5μ) | All the system suitability results are within the limit in Figure 7. |

Table 2: Matrix effects and extraction recoveries for Selexipag in rat plasma (n=6)

| Spiked concentration (ng/ml) | Matrix effect (%) (mean ± SD, n=6) | Recovery (%) (mean ± SD, n=6) |
|-----------------------------|------------------------------------|-------------------------------|
| 5.0                         | 97.85±5.05                         | 98.21±2.36                   |
| 10.0                        | 99.66±10.15                        | 99.89±6.22                   |
| 15.0                        | 98.63±8.38                         | 98.92±5.37                   |

Table 3: Linearity data for Selexipag calibration standards

| Linearity | Selexipag Conc. (ng/ml) | Selexipag Peak response | Internal Standard (IS) Peak response | Area ratio |
|-----------|-------------------------|-------------------------|-------------------------------------|------------|
| Linearity-1 | 1                      | 0.105                   | 1.021                               | 0.103      |
| Linearity-2 | 3                      | 0.254                   | 1.022                               | 0.249      |
| Linearity-3 | 5                      | 0.526                   | 1.026                               | 0.513      |
| Linearity-4 | 8                      | 0.754                   | 1.024                               | 0.736      |
| Linearity-5 | 10                     | 1.051                   | 1.027                               | 1.023      |
| Linearity-6 | 12.5                   | 1.259                   | 1.025                               | 1.228      |
| Linearity-7 | 15                     | 1.529                   | 1.021                               | 1.498      |
| Linearity-8 | 20                     | 2.055                   | 1.022                               | 2.011      |

Slope: 0.000980  
Intercept: 0.00273  
Correlation coefficient: 0.999

The drug substance Selexipag is developed by Actelion of oral treatment for selective IP receptor agonist. In fact, it can convert to an effective metabolite (Mubarak, 2010) by rapidly hydrolyzed in the presence of hepatic microsomes. Moreover, Selexipag (Figure 1) and its active metabolite (ACT-333679, 37-fold potent than parent analyte) are agonists (Kenakin, 2001) have a greater binding affinity for the human Prostacyclin receptor (IP receptor) compared with any other prostanoid.
Table 4: Inter-run and between-run precision and accuracy for Selexipag

| Nominal Conc. (ng/ml) | Inter run | Between run |
|----------------------|-----------|-------------|
|                      | Average (mean ± SD, n=6, ng/mL) | Precision (%CV) | Accuracy (%) | Average (mean ± SD, n=6, ng/mL) | Precision (%CV) | Accuracy (%) |
| 1.0                  | 0.99      | 1.05        | 98.2        | 5.02       | 1.04        | 97.3         |
| 5.0                  | 5.01      | 1.11        | 99.5        | 49.98      | 1.07        | 99.2         |
| 10.0                 | 10.01     | 1.01        | 100.1       | 10.11      | 1.00        | 100.05       |
| 15.0                 | 14.99     | 0.87        | 100.6       | 15.04      | 0.86        | 100.17       |

Table 5: Stability of the Selexipag samples

| Stability experiments                                      | Spiked plasma concentration (mean ± SD, n=6, ng/mL) | Concentration measured (mean ± SD, n=6, ng/mL) | %CV (n=6) |
|-----------------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-----------|
| Benchtop stability (for 24h)                              | LQC 1.0                                         | 0.98                                            | 1.5       |
| Autosampler stability (6°C for 24h)                       | HQC 15.0                                        | 15.02                                           | 0.7       |
| Long term stability (for 27-days)                        | LQC 1.0                                         | 1.01                                            | 1.2       |
| Freeze-thaw stability (for 24h)                           | HQC 15.0                                        | 14.99                                           | 1.5       |

Table 6: Mean pharmacokinetic parameters of Selexipag in rat plasma

| Pharmacokinetic Parameters | Selexipag |
|----------------------------|-----------|
| AUC0-t (ng h/ml)            | 3         |
| Cmax (ng/ml)                | 12.5      |
| AUC0-∞ (ng h/ml)            | 17        |
| Kel                         | 0.5       |
| t1/2                        | 1.38      |
| tmax (h)                    | 2.0       |

Table 7: Comparison of Selexipag % recovery between rat plasma, rabbit plasma, human plasma and rat urine

| Selexipag Extraction in Average recovery in % | Rat plasma | Rabbit plasma | Human plasma | Rat urine |
|----------------------------------------------|------------|---------------|--------------|-----------|
| Six replicates of HQC                        | 94.74      | 59.71         | 57.86        | 80.37     |
| Six replicates of MQC                        | 97.12      | 60.46         | 61.36        | 82.78     |
| Six replicates of LQC                        | 95.21      | 68.83         | 77.40        | 95.78     |
| Six replicates of LLQC                       | 94.68      | 55.40         | 70.42        | 91.16     |
Figure 10: Blank plasma chromatogram for Selexipag in rat plasma

Figure 11: LLOQ QC chromatogram for Selexipag in rat plasma

Figure 12: Calibration plot for Concentration v/s Area ratio of Selexipag and Internal Standard

Figure 13: Recovery plot for Selexipag in Rat plasma (Concentration in ng/mL Vs Time in Hours)

Figure 14: Blank plasma chromatogram for Selexipag in rabbit plasma

Figure 15: Blank plasma chromatogram for Selexipag in human plasma

Figure 16: Blank plasma chromatogram for Selexipag in rat urine

Figure 17: LLOQ QC chromatogram for Selexipag in rabbit plasma
Figure 18: LLOQ QC chromatogram for Selexipag in human plasma

Figure 19: LLOQ QC chromatogram for Selexipag in rat urine

receptor (Kuwano et al., 2007) leads to vasodilation (Costa and Biaggioni, 1998) in the pulmonary circulation (Sitbon and Morrell, 2012). Usage of Selexipag along with strong inhibitors of the liver enzyme such as gemfibrozil (Ogilvie, 2005) contraindicated in Europe. It is because the concentrations of Selexipag increased to twofold, which leads to active metabolite increased to 11-fold caused likely more inimical effects (Barst et al., 1996). In fact, selexipag and its active metabolite are chemically different from prostacyclin with peculiar pharmacology have similar modes of action to that of internal prostacyclin. Therefore, selexipag may be an alternative fascinating oral prostacyclin analogue for the treatment of PAH. The current proof-of-concept study was created to assess the safety, tolerability and efficacy of selexipag in adult patients with emblematic PAH. In fact, the inimical effects of Selexipag are similar to those of intravenous prostacyclins (Kermode et al., 1991) used for the treatment of PAH. However, in the usage of Selexipag drug, commonly occurred side effects are headache and jaw pain. Moreover, in people taking Selexipag also suffered a high risk of increased hyperthyroidism (Garber et al., 2012). By applying, an accurate and precise validated method for the treatment of PAH using Selexipag avoided or reduced side effects.

Ambrisentan was selected as internal standard, since this this drug can be used for PAH treatment (Figure 2). Bioanalysis is an area of analytical chemistry (Damireddy et al., 2017) in the quantitative measurement of xenobiotics (Gorumutchu and Ratnakaram, 2018; Patil et al., 2017; Ratnakaram, 2018) and biotics in biological systems. Bioanalytical common techniques reported in the literature are, hyphenated techniques like LC-MS, GC-MS, LC-DAD, CE-MS, chromatographic methods like HPLC, GC, UPLC, Supercritical fluid chromatography. Bioanalytical (Shankar et al., 2006) methods are generally deal with biological samples consists of the analyte along with a distinct range of chemicals that can have a conflicting impact on their accurate and precise quantifications. As such, a wide range of techniques reported extracting the analyte from its matrix such as protein precipitation, solid phase extraction and liquid-liquid extraction.

In this context, we are interested in developing simple, specific, accurate and precise various chromatographic and spectroscopic methods for validation of various drugs. For quantification of Selexipag drug one HPLC method (Damireddy et al., 2017) and three spectrophotometric methods were reported in the literature. However, no LC-MS method reported so far to the best of our knowledge. In this study, we report on the development and validation of an LC-MS/MS method for quantification of Selexipag in Rat plasma. Moreover, we also report the application for this LC-MS/MS method to its pharmacokinetics studies.

MATERIALS AND METHODS

Chemicals and Standards

The APIs of Selexipag (purity > 99%) and Ambrisentan (purity > 99%) were procured from Glenmark Pharmaceuticals, Mumbai. LCMS grade of acetonitrile was procured from J.T. Baker. HPLC grade of hexane sulfonic acid and orthophosphoric acid were procured from Merck and rat plasma was purchased from Bharat Biotech, Hyderabad.

Instrumentation

An instrument HPLC system (Waters Alliance e2695 model), connected with LCMS (QTRAP 5500 triple quadrupole) was used to develop the method and its validation. Empower 2.0 software was used for processing the data.

Detection

The LCMS instrument functioned in MRM (multiple reaction monitoring) modes and a +ve mode of electrospray ionization is used to sample introduction. The optimized conditions were as follows: the selected flow of nebulizer gas was 30 psi, the flow of curtain gas -25 psi, voltage of ion-spray 2000v
and the temperature was 375 °C. The potentials of declustering pressure (DP), focusing pressure (FP), entrance pressure (EP), collision energy (CE), cell exist (CXP) for Selexipag are 45, 35, 10, 8 ev and 12 respectively. The CAD (Collision activated dissociation) gas was adjusted to 4 psi with nitrogen gas. Both quadrupoles 1 and 3 were continued as single resolution and the time was 300 ms. The changes in mass were selected as m/z 462.5 to 496.2 for Selexipag and 378.5 to 402.6 for Ambrisentan respectively. The CAD (Collision activated dissociation) gas was adjusted to 4 psi with nitrogen gas. Both quadrupoles 1 and 3 were continued as single resolution and the time was 300 ms. The changes in mass were selected as m/z 462.5 to 496.2 for Selexipag and 378.5 to 402.6 for Ambrisentan respectively.

**Chromatography**

An analytical column of Waters Company, X-Bridge C18 (150 x 4.6 mm, 3.5 μm) was used in this analysis. Buffer was prepared with 2.5 g of hexane sulfonic acid in 1000mL water was adjusted to pH 2.5 by using orthophosphoric acid and mobile phase consists of buffer and acetonitrile in the ratio of 80:20 v/v, respectively. The source flow rate was 300 μl/min without split and the injection volume was 10 μl. Selexipag was eluted at 2.92 ± 0.2 min and total injection run time of 10 min. Selexipag and Ambrisentan were eluted at retention time of 2.9 min and 7.4 min respectively.

**Linearity graph for standards and QC samples**

Two types of stock solutions of Selexipag were prepared using acetonitrile as solvent. One stock solution was used for calibration standards in method validation and the second stock solution was used for QC sample analysis. By using first stock solution calibration curve was drawn at different concentrations of 1, 2.5, 5, 7.5, 10, 12.5, 15 and 20 ng/ml by spiking suitable quantity of Selexipag in blank rat plasma. Similarly, quality control samples at various concentrations 1, 5, 10 and 15 ng/ml of Selexipag were prepared using second stock solution.

**Extraction of plasma**

Rats were placed in metabolic cages for urine collection after for 2 hours and stored in refrigerator. Rat plasma and Rabbit plasmas were collected by cardiac puncture, and from liver. To collect plasma, used tubes that contains dipotassium ethylene diamine tetra acetate (K₂EDTA) as an anticoagulant (used 10 μl of 0.1M K₂EDTA for 200-400 μl of plasma; ensured the concentration of K₂EDTA used does not interfere with the downstream assay) and stored in refrigerator. Human plasma was collected in tubes that contain K₂EDTA as an anticoagulant and stored in refrigerator.

**Preparation of Spiked Sample solution in Rat Plasma**

The test sample was prepared by mixing of 500 μl of rat plasma, 500 μl of acetonitrile, 500 μl of internal standard (IS) and 500 μl of the sample. The samples were precipitated from the respective solutions using vortex cyclo mixture for 10 minutes. Centrifuged at 4000 rpm for 20 mins. Later collected the supernatant solution in HPLC vial and injected into the chromatographic system.

**Preparation of Spiked Sample solution in Rabbit Plasma**

The test sample was prepared by mixing of 500 μl of rabbit plasma, 500 μl of acetonitrile, 500 μl of internal standard (IS) and 500 μl of the sample. The samples were precipitated from the respective solutions using vortex cyclo mixture for 10 minutes. Centrifuged at 4000 rpm for 20 mins. Later collected the supernatant solution in HPLC vial and injected into the chromatographic system.

**Preparation of Spiked Sample solution in Human Plasma**

The test sample was prepared by mixing of 500 μl of human plasma, 500 μl of acetonitrile, 500 μl of internal standard (IS) and 500 μl of the sample. The samples were precipitated from the respective solutions using vortex cyclo mixture for 10 minutes. Centrifuged at 4000 rpm for 20 mins. Later collected the supernatant solution in HPLC vial and injected into the chromatographic system.

**Preparation of Spiked Sample solution in Rat Urine**

The test sample was prepared by mixing of 500 μl of human plasma, 500 μl of acetonitrile, 500 μl of internal standard (IS) and 500 μl of the sample. The samples were precipitated from the respective solutions using vortex cyclo mixture for 10 minutes. Centrifuged at 4000 rpm for 20 mins. Later collected the supernatant solution in HPLC vial and injected into the chromatographic system.

**Selectivity**

In plasma selectivity measurements six different lots in rat plasma were taken and analyzed in three different concentrations such as high, medium and low level of quality control samples.

**Effect of Matrix**

Effect of the matrix in Selexipag along with IS was evaluated by relating the area of the peak ratio of the extracted plasma sample which is taken from six different rats. The tests were carried out at middle quality control (MQC) levels in three replicates with six different plasma lots within the acceptable range i.e., % CV ≤ 15 %.

**Accuracy and Precision**

It was obtained by the analysis of six QC samples at LLOQ QC, LQC, MQC and HQC levels. The acceptance
criteria of % CV should be below 15 % for precision and accuracy should be within 15 % except for LLOQ QC, which has 20 % sample accuracy.

**Recovery**

The effectiveness of extraction of Selexipag was determined by analysis of each quality control sample for six replicates. By relating the peak areas of extracted standards and non-extracted standards percentage recoveries were estimated for Selexipag.

**Stability**

Stability analysis was performed by relating the peak area ratio for internal standard in stability sample and freshly prepared sample solution. Stability analysis was carried out for six preparations of LQC and HQC concentration levels. As per US FDA guideline (Shankar et al., 2006; FDA, 2018) samples were stable below 15 %. Benchtop stability samples and 2-8°C in autosampler tested up to 24 hrs. Freeze-thaw stability samples were frozen at -30°C and thawed three times compared to a freshly prepared quality control sample. Six different samples of HQC and LQC concentration levels were used for freeze-thaw stability. Later they were stored at below 10°C to wet extracted stability samples for 24 hrs and compared the stability samples data with initially prepared fresh solutions data to assess the long term stability.

**RESULTS AND DISCUSSION**

**Method Development**

In the method development process, different types of techniques were optimized in the mass spectrometry such as chromatography and extraction of the samples. Different trails were performed as shown in Table 1 and their related figures are shown in Figures 3, 4, 5, 6 and 7.

**Optimization of Mass spectrometry detection parameters**

The high response electrospray ionization (ESI) was selected for this study. The optimization of the instrument to give signal stability and sensitivity during infusion of the sample analyte in the prolonged flow rate of mobile phase to electrospray ion source maintained at both polarities. Selexipag gives a high response in +ve ion mode when compared to –ve ion mode. The major peaks in the primary ESI spectra of Selexipag of MH+ ions observed at m/z 462.5 and 496.2, respectively (Figure 1). Internal standard Ambrisentan MH+ ions were observed at 378.5 and 402.6, respectively (Figure 2).

**Chromatography Optimization**

Different types of mobile phases were used to develop the present methods presented in Table 1. The pKa value of Selexipag was found to be 3.77. In acidic buffers, sharp peaks were obtained. In all the trails only acidic buffers were used for method development. Plate count and tailing factor for Selexipag were not satisfactory with the mobile phase composition of Buffer (0.1 % triethylamine pH adjusted to 2.5 with orthophosphoric acid) and acetonitrile in the ratio of 20:80 v/v. After that a mobile phase combination of hexane sulfonic acid/acetonitrile with the change in the composition was utilized for method development. Finally, the mobile phase composition of buffer (2.5 g hexane sulfonic acid in 1000 mL water with pH adjusted to pH 2.5 with orthophosphoric acid) and acetonitrile in the ratio of 80:20 v/v in gradient program was optimized. Later, an improvement in peak shape and peak response for Selexipag and internal standard was observed using the finalized mobile phase composition. An analytical column X-Bridge C18 (150 x 4.6 mm, 3.5 μm), Waters Company was finalized with a flow of 1.0 mL/min was used. The main analyte and internal standard were eluted within 10 min of run time (Table 1).

**Optimization of Sample Extraction**

Sample was extracted for removal of proteins from the prepared solution. At first tested with various extraction procedures like protein precipitation (PPT), liquid-liquid extraction (LLE) and solid phase extraction (SPE). In solid phase extraction (Figure 8) stationary phase made up of packed syringe-shaped cartridge, with 96 well plate and 47 mm flat disk packed with sorbent material in liquid handling syringe. Suppression effect was observed in protein precipitation method for internal standard and drug. In solid phase extraction, low analyte recovery was observed (80.56%). In all the extraction procedures Precipitation extraction is suitable for extraction of the internal standard and Selexipag drug. Various organic solvents such as ethyl acetate, acetonitrile, chloroform, n-hexane, dichloromethane and methyl tertiary butyl ether individually or as well as with combination in PPT were used to extract the analyte from plasma sample. Among them acetonitrile produces appreciable extraction results by using Ambrisentan as internal standard for this analysis. Moreover, there is no significant effect in internal standard on analyte recovery, sensitivity or ion suppression. However, in Protein Precipitation method (Figure 9), shows prominent recovery (92.64 %) and selectivity was observed.

Also observed appreciable recovery was observed
while extracted in Rat plasma compared to Rabbit plasma, Human plasma and Rat urine in protein precipitation method. These optimized parameters of the extracted procedure and chromatographic conditions bring about in reduced analysis time with accurate and precise detection of Selexipag in rat plasma.

**Method validation**

Validation of the analytical method for Selexipag assay in rat plasma was performed by as per ICH guidelines. This method was validated for the effect of the sample matrix, sensitivity, selectivity, accuracy, linearity, preciseness, reproducibility, and stability with high recoveries.

**Sensitivity and Selectivity**

Blank and spiked sample with a lower limit of quantification quality control (LLOQ QC) in rat plasma for Selexipag are shown in Figure 10 and Figure 11. The % interference of retention time of Selexipag peak was found to be 0.0% among six various batches of rat plasma, comprising hemolyzed and lipemic plasma containing K2EDTA as an anticoagulant, and it is in acceptance criteria. Six replicates of Selexipag extracted samples at the LLOQ QC level in one of the plasma sample having the least interference at the Selexipag retention time were analyzed. The % coefficient of variance for the peak is the response for six sample replicate was found to be 1.2 % for Selexipag. Similarly, blank and spiked sample with a lower limit of quantification quality control (LLOQ QC) in rabbit plasma, human plasma and rat urine for Selexipag are shown in Figures 14, 15, 16, 17, 18 and 19.

**Effect of Matrix**

For ion suppression/enhancement the % CV for the signal response was observed 1.0 % for MQC level for Selexipag, resembles that the effect of matrix on the ionization of analyte is well within the acceptable range under optimized conditions (Table 2).

**Linearity**

The Selexipag area ratio was proportional to the concentration of Selexipag prepared and injected for calibration standards. The Selexipag assay was determined over the concentration range of 1 to 20 ng/mL of Selexipag analyte. The calibration curves show linearity and represented in Figure 12. The correlation coefficient was ≥ 0.999 (Table 3) for various concentration levels of Selexipag.

**Accuracy and Precision**

Accuracy and precision were evaluated by mixing all separate assay results obtained of six replicates in four different days for quality control samples. The % CV for precision was < 5 % and accuracy was obtained in a range of 98 to 101 for Selexipag in all the days (inter-run). All the outcome of this data tabulated in Table 4 and shows the method accuracy and precision.

**Recovery**

Six aqueous spiked sample solutions at three different concentrations low, medium an high-quality control levels for Selexipag were prepared for recovery estimation, and the peak areas occurred for extracted samples of the same strength level from a precision and accuracy samples on the same day. The average recovery for Selexipag was 98.2 % with a precision of 1.2 %. This specifies that the extraction efficiency for Selexipag was reliable and reproducible.

Also found reproducible recoveries were observed for Selexipag in rat plasma while in comparison with rabbit plasma, human plasma and rat urine (Table 7).

**Reinjection Reproducibility**

This test was carried to verify whether the instrument performance remains unaffected due to any instrument failure during sample analysis. The change was less than 2.0 at LQC and HQC concentration levels at initial and even after analyzed 24 hr shows less % change and hence sample can analysis in case of instrument breakdown.

**Stability Studies**

In solution stability analysis, Selexipag in solutions were prepared in diluent and put in storage at 2-8°C in a refrigerator. Fresh stock solutions were related to aged stock solutions prepared earlier 24hr. The % change for Selexipag was 1.04 %, which shows that stock solutions were stable at least 24hr when stored in 2-8°C. Selexipag samples in bench top and instrument autosamplers were analyzed at HQC and LQC levels. Selexipag was stable in plasma for at least 24hr when stored at room temperature and 24hr in an autosampler when stored at 20°C. Hence it indicates that repeated freezing and thawing (three cycles) of samples in plasma spiked with Selexipag at HQC and LQC levels doesn’t impact their solution stability. The stability analysis results are tabulated below (Table 5) specifies that Selexipag was stable in a matrix up to 24hr at a storage condition of -30°C.

**Application to pharmacokinetic study**

The validated method has been successfully quantified the Selexipag concentration in various three groups of rats, under fasting conditions after administration of 800 mcg Selexipag drug sample was
injected into a rat body collected the samples at different time intervals like 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 hours. After that samples were prepared as specified method and injected in the chromatographic system and recorded the results. The pharmacokinetics parameters assessed were \( C_{\text{max}} \) (maximum observed drug concentration during the study). The values of AUC \(_{0-12}\) (area under the plasma concentration with time curve measured 2.0hr, using the trapezoidal rule), \( T_{\text{max}} \) (time to observed maximum drug concentration), \( K_{\text{el}} \) (apparent first order terminal rate constant calculated from a semi-log plot of the plasma concentration versus time curve, using the method of the least square regression) and \( t_{1/2} \) (terminal half-life as determined by the quotient 0.693/\( K_{\text{el}} \)) are tabulated in Table 6.

The test/reference ratios for \( C_{\text{max}} \), AUC \(_{0-12}\), and AUC were 80.56, 90.12 respectively, and they were within the acceptance range of 80%-125% demonstrating the bio-equivalence of the formulation of Selexipag (Figure 13).

**CONCLUSIONS**

The present proposed developed and validated quantification method shows high sensitive LC-ESI-MS/MS method for the estimation of Selexipag in rat plasma. Moreover, this method is fast, rugged, reproducible bio analytical method to be developed LC-ESI-MS/MS method for the estimation of Selexipag in rat plasma. The method was developed to assess the lower limit of detection (0.5 ng/mL) and limit of quantification (5 ng/mL) concentrations in rat plasma. Linearity was observed in calibration curves for assay Selexipag in a range of 1 ng/mL to 20 ng/mL of concentration in rat plasma for each analyte and its regression correlation coefficient was found to be 0.999. This analytical method is simple and well-organized and can be utilized in pharmacokinetics studies and also for monitoring of samples injected in body fluids too. We strongly believe that, this study can provide a road map for developing various new quantification methods for various drug molecules.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical department details for animal studies**

Registration No. Nirmala college of pharmacy, 1629/P0/a/12/CPCSEA.

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**Abbreviations**

LOQ: Low Quality control; HQC: High Quality control; MQC: Medium Quality Control; PPE: Protein precipitation extraction; LLE: Liquid liquid extraction; \( T_{\text{max}} \): Time required for \( C_{\text{max}} \); Min: Minutes; ng/mL: Nano gram per milli liter; RSD: Relative standard deviation; ICH: International council for harmonization; LOQ: Limit of quantitation; LOD: Limit of detection; LLQC: Low level Quality control.

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