Fluorimetric Derivatization-Based HPLC-FL Method for the Prototype Pharmacokinetic Analysis of Selexipag in Human Plasma

Burhan Ceylan†*, Meltem Çayci‡, Cem Önal§, Armağan Önal‖

† Harran University, Faculty of Pharmacy, Department of Pharmacognosy, Sanliurfa, Turkey;
*e-mail: b.ceylan022@gmail.com
‡ Harran University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, Sanlıurfa, Turkey;
§ Istanbul Health and Technology University, Faculty of Pharmacy, Department of Analytical Chemistry, Istanbul, Turkey;
‖ Istanbul University, Faculty of Pharmacy, Department of Analytical Chemistry, 34116 Beyazit-Istanbul, Turkey

Received: February 18, 2025; Accepted: April 24, 2025
DOI: 10.17721/moca.2025.117-122

A simple and cost-effective HPLC-FL method has been developed for measuring selexipag in human plasma, showcasing its suitability for pharmacokinetic research. Selexipag was precolumn derivatized with 7-chloro-4-nitrobenzofurazan (NBD-Cl) and the fluorescent derivative was separated on a C18 (150 mm × 4.6 mm × 2.6 μm) analytical column at 30 ºC using a mobile phase composed of acetonitrile – 0.1% o-phosphoric acid in water (70:30, v/v) by isocratic elution with flow rate of 1.0 mL min^{-1}. The method was based on measuring the derivative using fluorescence detection (λ_{ex} = 380 nm, λ_{em} = 420 nm). The retention time of selexipag is 6.40 ± 0.01 min. This currently developed method was validated according to EMA criteria by evaluating the specificity, linearity, precision, accuracy, and robustness. The method was determined to be linear in a concentration range of 0.01-20 ng mL^{-1} with a correlation coefficient of 0.9998. LOD and LOQ were found to be 0.003 and 0.01 ng mL^{-1}, respectively. Intraday and interday RSD values were less than 1.75%. The plasma concentration-time profile and pharmacokinetic parameters such as AUC_{0-t}, $AUC_{0-\infty}$, C_{max}, t_{max}, $t_{1/2}$, were calculated according to the assays. The presented method can be effectively used for bioequivalence and bioavailability investigations, as well as for routine analysis of the drug in plasma.

Keywords: selexipag, HPLC-FL, pre-column derivatization, pharmacokinetics, NBD-Cl

Introduction

Pulmonary arterial hypertension (PAH) is a medical condition where the pressure in the arteries that carry blood from the heart to the lungs becomes abnormally high. This happens because their arteries become narrowed, forcing the heart to work harder to circulate blood. As a result, individuals with PAH often experience symptoms like extreme tiredness, lightheadedness, and difficulty breathing. Selexipag (SLP) is used for the ongoing treatment of PAH in adults whose condition is not well managed by other PAH medications, including endothelin receptor antagonists or phosphodiesterase type 5 inhibitors. It helps relax and widen the pulmonary arteries, mimicking the effects of prostacyclin [1]. By easing the heart's workload in pumping blood through the pulmonary arteries, SLP helps lower the pressure within them. This not only relieves the symptoms of PAH but also helps slow down the advancement of the disease [2,3]. The chemical structure of SLP is represented by the formula 2-(4-((5,6-diphenylpyrazin-2-yl)(isopropyl)amino)butoxy)-N-(methylsulfonyl) acetamide, as illustrated in Figure S1.

The available literature lacks studies on the use of the HPLC-FL method for measuring SLP in human plasma. Moreover, there are no reliable and straightforward methods for conducting pharmacokinetic studies to assess SLP concentrations in human plasma. Various methods have been used to measure SLP in the literature, including visible spectrophotometry [4], gas chromatography [5], and HPLC for both bulk substances and pharmaceutical products [6]. Furthermore, two distinct studies employed LC-MS/MS and UPLC-MS/MS techniques to analyze the compound in rat plasma [7,8]. An HPLC-UV method is available for measuring SLP in human plasma [9,10]. Current liquid chromatography methods are disadvantaged by their expensive detector requirements, low sensitivity, and long sample preparation procedures. The liquid chromatography method combined with the pre-column derivatization procedure we developed has the highest sensitivity and selectivity among all the methods developed for the quantification of selexipag.

This study was designed to quantify SLP levels in human plasma using an HPLC-FL technique, involving derivatization with NBD-Cl. The novel HPLC-FL approach was effectively applied to evaluate the pharmacokinetic profile of SLP tablets. The pharmacokinetic analysis was conducted using

plasma samples from a healthy volunteer, with approval from the ethics committee, following the administration of 800 μg of SLP.

Experimental part

Chemicals and reagents

Shanghai Yingxuan Pharmaceutical Science & Technology (China) provided selexipag, while the local pharmacy sold Uptravi film-coated tablets with 800 μg of selexipag. The following were provided by Merck (Darmstadt, Germany): acetonitrile (HPLC grade), orthophosphoric acid (HPLC grade), monobasic dihydrogen phosphate and dibasic monohydrogen phosphate, hydrochloric acid (analytical grade), and chloroform (analytical grade). The source of NBD-Cl was Sigma Aldrich, located in St. Louis, USA. A Human (Japan) ultrawater filtration system was used to purify the water.

Solutions

Standard solutions ranging from 0.01 to 20 ng mL^{-1} were prepared by diluting a stock solution of selexipag (100.0 μg mL^{-1}) in acetonitrile. In 50 mL of water, 2.0209 grams of sodium phosphate dibasic and 0.3394 grams of sodium phosphate monobasic solution were combined to create phosphate buffer. A 0.1 M hydrochloric acid solution was used to adjust the pH level to 8.5, and 100 mL of water was added to bring the volume up to 100 mL. A new NBD-Cl solution containing 5 mg mL^{-1} of methanol was prepared. The other solutions remained stable for more than two weeks while being kept at 4 ºC.

Instrumentation and chromatographic conditions

Using a Shimadzu spectrofluorimeter Model RF-1501 fitted with a xenon lamp and 1 cm quartz cells, fluorescence spectra and measurements were obtained. The wavelengths for excitation and emission were chosen at 380 and 420 nm, respectively. We used a WTW pH 526 digital pH meter to measure the pH. A Shimadzu (Japan) LC-20 liquid chromatograph, which included a CTO-10 AC column oven, a SIL-20AT-HT autosampler component, an SPD-20A HT, and an LC-20AT pump, was used to conduct the HPLC analyses.

To achieve the most effective chromatographic separation, several combinations of mobile phase, column types, and stationary phase size were tested with varying flow rates and column temperatures. With a C18 Inertsil® (150 × 4.6 mm × 2.6 μm) analytical column at 30 ºC, chromatographic separation was accomplished isocratically using a mobile phase consisting of acetonitrile-0.1 % o-phosphoric acid in water (70:30, v/v) at a flow rate of 1.0 mL min^{-1}.

Sample preparation and general procedure

Five milliliters of venous blood were drawn from a volunteer's peripheral veins (an informed consent form was obtained with approval from the ethical committee), placed in tubes containing disodium EDTA, and centrifuged for 10 minutes at 4500 × g. The resulting plasma samples were stored at -20 degrees Celsius. SLP was extracted from the plasma samples by alkalinizing 1.0 mL of plasma with 500 μL 0.1 M NaOH, adding selexipag working solutions, and then extracting the solution into 5 mL of chloroform. After five minutes of moderate-speed vortex mixing, the contents were centrifuged for five minutes at 4500 × g.

The watery layer was thrown away. At 40 ºC, a spray of nitrogen was used to evaporate the organic layer until it was completely dry. After adding 500 μL of pH 8.5 phosphate buffer, 500 μL of 5 mg mL^{-1} NBD-Cl solution, and 1 mL of water to the residue, the system was heated for seven minutes at 80 °C. After cooling in an ice bath, 0.2 mL of 1 N HCl solution was used to acidify the mixture. After 30 seconds of vigorous mixing the solution with a vortex mixer, 20 μL of the derivatized sample was introduced into the HPLC apparatus.

Results and discussion

Derivatization process

The conditions under which selexipag and NBD-Cl reacted were examined and improved for the SLP-NBD-Cl derivative efficiency. Every parameter has been altered independently, while the others remained unchanged. The ideal reaction time, temperature, pH, type of buffer, acetonitrile-water proportions, molar ratio of NBD-Cl to selexipag, concentration of HCl, and amount of acidification required to halt the derivatization reaction were all established. Figure 1 illustrates the response of derivatization.

Effect of pH

NBD-Cl is a nonfluorescent reagent that reacts with amino or thiol groups to become highly fluorescent. Furthermore, NBD-Cl offers a straightforward and accurate technique for identifying N-terminal amino acids. Prolyl peptides can be identified by exploiting variations in fluorescence color and intensity [11]. The

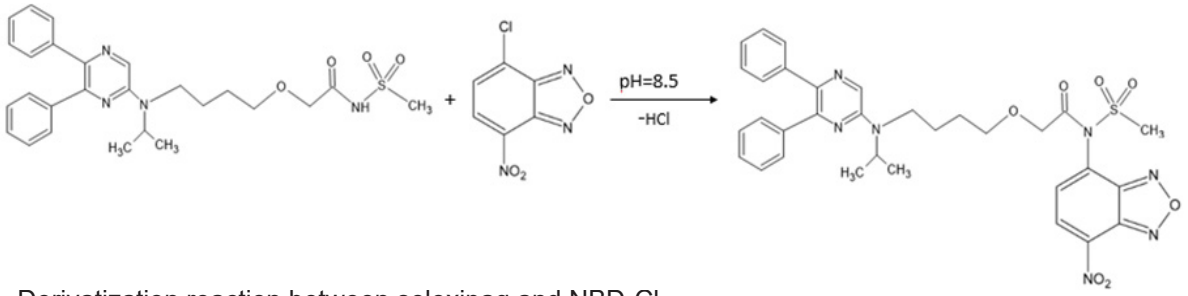

Fig. 1. Derivatization reaction between selexipag and NBD-Cl.

research on pH was limited to the range of 7–11 using phosphate buffer, as it was observed that fluorescence emission was only created in alkaline media [12]. At a pH of 8.5, the maximum absorbance of the suggested method was achieved. Chromatogram (Figure S5) and graph (Figure S8) showing the effect of pH change on the peak area and the optimum pH and presented in the supplementary material section.

Effect of time and temperature

The derivatization reaction was conducted at various temperatures and times to ascertain the ideal temperature and reaction duration. The mixture was heated in a thermostatically controlled water bath at 80 ºC for 7 minutes to create the fluorophore. The temperature values used for optimization range from 30 to 100 °C. Chromatogram (Figure S6) and graph (Figure S9) showing the effect of temperature change on the peak area and the optimum temperature and presented in the supplementary material section.

Effect of NBD-Cl concentration

The study investigated the effect of NBD-Cl concentration on the derivatization reaction. 0.025 mmoL (500 µL of 0.5% (w/v)) NBD-Cl solution was determined to be adequate to achieve the highest intensity. The concentration of NBD-Cl used for optimization ranges from 0.010 to 0.040 mmoL. Chromatograms (Figure S4) and graph (Figure S7) showing the effect of NBD-Cl concentration on the peak area and the optimum NBD-Cl concentration are presented in the supplementary material section.

Effect of acetonitrile to water ratio in derivatization medium

Various acetonitrile and water volumes were tested while maintaining constant concentrations of medication, buffer, and NBD-Cl solution. Using a 1:3 acetonitrile to water ratio produced maximum peak area.

Stoichiometry of the reaction

Using Job's method of continuous variation, the molar ratio of NBD-Cl to selexipag in the reaction mixture was examined [13]. The reaction stoichiometry was determined to be approximately 1:1 using equimolar solutions of selexipag and NBD-Cl. Peak regions indicate that there is neither a shortage nor an excess of the reagent in this stoichiometric ratio, indicating that all of the reagent has been utilized. To determine the ideal conditions for investigating derivatization reactions, all solutions were injected into an HPLC machine, and peak areas were measured. For at least 24 hours, derivatives prepared under the aforementioned circumstances stayed stable.

Effect of HCl concentration for acidification

The most effective results were obtained with 0.2 mL of 1.0 N HCl to generate NBD-OH, thereby eliminating the excess NBD-Cl.

In the optimization processes in the derivatization procedure, all measurements were repeated 3 times, and SD and RSD values were given in the accuracy and precision studies.

Chromatographic process

Using an isocratic elution technique and HPLC-FL, as previously mentioned, a satisfactory separation of the derivatives and endogenous chemicals of plasma was achieved. Figure S2 a-e shows representative chromatograms of the blank solution, standard solution, blank plasma, spiked plasma, plasma samples spiked with 10 ng mL^{-1} selexipag, and plasma samples of the volunteer who took Uptravi® film-coated tablets containing 800 µg selexipag at t_{max}. There was no evidence of interference with the SLP of the plasma. Selexipag has a retention time of 6.40 ± 0.01 minutes. The suitability parameters of the chromatographic system are shown in Table S1. Chromatograms are necessary to determine the optimization of derivatization conditions (pH, temperature, NBD-Cl) and are presented in the supplementary material section. Not all these additional chromatograms show good resolution.

Validation of the Method

Following the guidelines provided by the European Medical Agency (EMA), the procedure was validated [14].

Linearity and sensitivity

Using a calibration curve in the range of 0.01–20 ng mL^{-1} of the SLP (n=5), the method's linearity was assessed. Five milliliters of selexipag plasma samples, spiked with varying quantities of each working standard selexipag solution, were analyzed to create calibration curves. Following that, the samples were put through the previously mentioned extraction, derivatization, chromatographic separation, and fluorometric detection procedures. Plotting the peak regions of the derivative against the corresponding selexipag concentrations enabled the creation of calibration curves using linear least-squares regression analysis. The calibration curve (n=5) derived from five points (0.01, 0.1, 1.0, 10.0, 20.0 ng mL^{-1}) has the following equation: $y = 342606\ x + 19135$ (correlation coefficient = 0.9998), where x is the selexipag concentration and y is the peak area of the selexipag-NBD-Cl derivative.

LOD or LOQ = kSDa/b, where k=3 for LOD and 10 for LOQ, was used to calculate the limit of detection (LOD) and limit of quantitaion (LOQ). SDa is the standard deviation of the intercept, and b is the slope. Table 1 provides a summary of the parameters for the analytical performance of the suggested approach. The biggest advantage of the method we developed, compared to other liquid chromatography methods, is that it enables selective analysis of the active ingredient at ng/mL level in such a sensitive concentration and complex sample environment.

Accuracy, precision and recovery

QC samples were determined at three concentration levels in order to evaluate accuracy and precision. QC samples were created in plasma and aqueous samples at three distinct concentrations (0.01, 1.0, and 20 ng mL^{-1}) that fall into the low, medium, and high concentration (n=3) categories. Recovery values

and RME were used to express accuracy, whereas RSD was used to express precision. By extracting and derivatizing selexipag-spike plasma samples and comparing the peak regions obtained after derivatizing the same amounts of aqueous unextracted selexipag solutions, the 100% recovery of selexipag from plasma samples was investigated. Selexipag's mean absolute recovery was 99.38%. By comparing the amounts that are added to spiked and measured by the calibration curve, the mean relative recovery was determined to be 98.96%. For intraday precision and accuracy, three replicates of each concentration were assayed on the same day, and for interday precision and accuracy, three separate days were used. All of the intraday and interday assays had RDS values below 1.75%. Based on all of these findings, which are compiled in Table 2, good accuracy and precision were noted.

Table 1. Analytical parameters of the method.

Parameters	Method
Concentration range [a] (ng mL^{-1})	0.01-20
Regression equation[b]	y=342606x+19135
Intercept±SD	342606±177
Slope±SD	19135±28
Correlation coefficient (r)	0.9998
LOD (ng mL^{-1})	0.003
LOQ (ng mL^{-1})	0.01

[a] average of six determinatons; [b] $y=xC$ +b where C is the concentration in ng mL^{-1} and y is the peak area.

Robustness

As mentioned in the validation section above, robustness was evaluated by determining the QC samples at three concentration levels (n=3). The flow rate, column oven temperature, and the amount of acetonitrile and water phase in the mobile phase are the variables that are altered to gauge how robust the procedure is. Changes were made to the column temperature from 30 ºC to 25 ºC and 35 ºC, the proportions of the mobile phase (acetonitrile-water solution) from 70:30 v/v to 75:25 and 65:35, and the flow rate from 1.0 mL min^{-1} to 0.9 and 1.1 mL min^{-1}. Peak area and resolution were not significantly impacted by these adjustments. Low RSD values demonstrate the method's resilience in Table 3.

Stability

Three replicates and various storage settings were used to examine the stability of working standard selexipag solutions at QC levels. The experimental storage settings include 24 hours of dark, room temperature storage, 24 hours of autosampler conditions, and 1 month of refrigeration at 4 ºC. The recovery rates for the conditions under testing are 97.6%, 98.8%, and 98.1%, respectively. The recovery percentage numbers are higher than those from our earlier research [15]. Out of all these studies, the highest RSD percentage was 2.48%. It should be noted that selexipag was shown to be stable under all test setting.

Table 3. Robustness of the method.

Condition	Value	Recovery %	RSD %
Flow rate mL min^{-1}	0.9	99.3	0.67
	1.1	99.1	0.72
Mobile phase composition (methanol: aqueous phase)	65:35	97.9	2.01
	75:25	98.1	1.96
Column temperature	25	99.1	0.41
	35	99.3	0.43

n=3 for all QC sample levels

Application of the Method to Pharmacokinetic Analysis

In order to determine the SLP material in plasma for the pharmacokinetic research, the proposed approach was used. Selexipag (800 µg) was administered orally as a single dose to a healthy 35-year-old male volunteer. On the first day, approximately 5 mL of venous blood samples were taken before the dosage and at 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 7, 8, and 12 hours after the dosage. During the next five days, blood samples were taken once daily. As previously mentioned, the blood samples were converted to

Table 2. Accuracy and precision of the method.

Existant concentration (ng mL^{-1})	Added concentration (ng mL^{-1})	Found concentration (ng mL^{-1}) (Mean±SD[1])	Recovery (%)	RSD of recovery	RSD of intraday variation	RSD of interday variation
1	0.01	1.00 ± 0.01	99.00	1.18	0.98	1.40
	1	1.97 ± 0.04	98.50	1.82	1.24	1.75
	20	20.87 ± 0.02	99.38	1.02	0.80	1.26
Mean relative recovery = 98.96						

For each concentration n=3

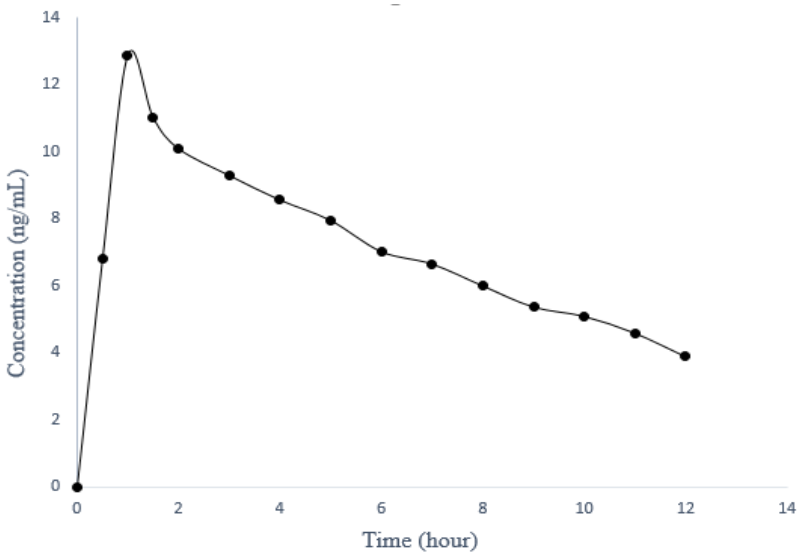

Fig. 2. Pharmacokinetic curve of selexipag after administration of 800 µg dose orall.

plasma. A chromatogram of the plasma sample taken 1.0 hours after the volunteer received a single oral dose of 800 µg selexipag is displayed in Figure 3e. Until analysis, the samples were kept at -20 ºC.

Using the analysis performed according to the suggested strategy, pharmacokinetic parameters were calculated. The TOPFIT 2.0 pharmacokinetic and pharmacodynamic data analysis system was used to compute the area under the plasma concentration-time curves (AUC_{0-12}, $AUC_{0-\infty}$) [16]. Figure 2 displays the selexipag plasma concentration-time curve following the oral administration of a single 800 µg dose of medication. The pharmacokinetic parameters listed in Table 4 are the same as those that were discovered earlier [9].

Table 4. Pharmacokinetic parameters of selexipag after administration of single oral dose of 800 µg.

Parameter	Found value
Time to maximum concentration T_{max} (h)	1.0
Maximum concentration C_{max} (ng mL^{-1})	12.8
Elimination half life $t_{1/2}$ (h)	6.8
AUC^{d}_{0-12} (ng h mL^{-1})	221.3
$AUC^{d}_{0-\infty}$ (ng h mL^{-1})	240.7

Conclusion

The developed HPLC-FL method provides a robust and practical tool for therapeutic SLP monitoring, especially in clinical research setting where sensitivity and cost-efficiency are critical. SLP is a relatively novel active substance for the treatment of pulmonary arterial hypertension. Since this SLP-NBD-Cl is relatively new, it is important to conduct further research on possible interactions with other medications and food, as well as its side effects. The HPLC-FL technique presented here is reliable, highly sensitive, and cost-efficient. With a retention time of around 6.40 minutes, the analysis is completed quickly. Almost any laboratory can use the highly fluorescent selexipag derivatives with NBD-Cl for precise fluorometric detection of the drug using simple HPLC-FL. This is the first time selexipag has been identified using fluorometric methods in published research. This method is well-suited for routine analysis of the drug in plasma, as well as for bioequivalence and bioavailability assesments.

Authors' Contributions

All the authors contributed equally. The author(s) read and approved the final manuscript.

Declarations

Conflicts of Interest/Competing Interests

The authors declare no conflicts of interest/ competing interests.

Ethical Approval

All procedure performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Bezmialem Vakıf University approved by the Clinical Trials Ethic Commitee (No: 2022/33).

Informed consent

Informed consent was obtained from all individual participants included in the study.

References

1. Richter, M. J.; Gall, H.; Grimminger, J.; Grimminger, F.; Ghofrani, H-A. Selexipag for the treatment of pulmonary arterial hypertension. *Expert Opin Pharmacother.* 2016, *17* (13), 1825-1834. https://doi.org/10.1080/14656566.2016.1215429

2. Simonneau, G.; Torbicki, A.; Hoeper, M. M.; et al. Selexipag: an oral, selective prostacyclin receptor

agonist for the treatment of pulmonary arterial hypertension. *Eur. Respir. J.* 2012, *40* (4), 874-880. https://doi.org/10.1183/09031936.00137511

3. Kaufmann, P.; Hurst, N.; Astruc, B., Dingemanse, J. Absolute oral bioavailability of selexipag, a novel oral prostacyclin IP receptor agonist. *Eur. J. Clin. Pharmacol.* 2017, *73* (2), 151-156. https://doi.org/10.1007/s00228-016-2164-4

4. Gorumutchu, G. P.; Ratnakaram, V. N. Oxidative Coupling: A tranquil approach for determination of selexipag by visible spectrophotometry. *Orient. J. Chem. 34* (6), 3112-3117. http://dx.doi.org/10.13005/ojc/340656

5. Li, L.; Wang, H.; Tian H.; Li, J., et al. Determination of 6 residual solvents in selexipag by headspace gas chromatography. *J. China Pharma. Univ.* 2018, *49* (1), 93-96. http://dx.doi.org/10.11665/j.issn.1000-5048.20180113

6. Santosh, S. S.; Devi, N.K. Stability indicating method development and validation of selexipag in bulk and pharmaceutical dosage form by using RP-HPLC. *J. Pharm. Res.,* 2021, *5* (2), 000239. https://doi.org/10.23880/oajpr-16000239

7. Xie, S.; Shi, L.; Chen, J.; et al. Simultaneous quantification and pharmacokinetic investigation of selexipag and its main metabolite ACT-333679 in rat plasma by UPLC-MS/MS method. *J. Pharm. and Biomed. Anal.* 2020, *190,* 113496. https://doi.org/10.1016/j.jpba.2020.113496

8. Rao, K. P.; Babu, N. L.; Koganti, K.; et al. Related substances method development and validation of an LC-MS/MS method for quantification of selexipag and its related impurities in rat plasma and application to pharmacokinetic studies. *SN App. Sci.* 2021, *3,* 3, 321. https://link.springer.com/article/10.1007/s42452-021-04219-x

9. Ceylan, B.; Tırıs, G.; Tekkeli Kepekci, S. E.; et al. A novel HPLC method for selexipag in human plasma and application to a prototype pharmacokinetic study. *Acta Chrom.* 2024, *36,* 3, 273-278. https://doi.org/10.1556/1326.2023.01167

10. Satheshkumar, V.; Muruganantham, V. A rabit method for quantification of selexipag in human plasma using high performance liquid chromatography with electron spray ionzationtendem mass spectrometry. *Annals R.S.C.B.* 2021, *25,* 4, 5689-5707.

11. Fager, R. S.; Kutina, C. B.; Abrahamson, E. W. The use of NBD chloride (7 chloro-4-nitro-2-oxa-1,3-diazole) in detecting amino acids and as an N-terminal reagent. *Anal Biochem.* 1973, *53,* 1, 290-294. https://doi.org/10.1016/0003-2697(73)90433-8

12. Tekkeli Kepekci, S. E.; Önal, A. Spectrofluorimetric methods for the determination of gemifloxacin in tablets and spiked plasma samples. *J. Fluoresc.* 2011, *21,* 1001-1007. https://doi.org/10.1007/s10895-010-0759-1

13. Job, P. Study of new selective reagent acetophenone 2',4'- Dihydroxy semicarbazone for extractive spectrophotometric determination of vanadium. *Ann. Chim.* 1928, *9,* 113-203.

14. EMA (2011). European Medicines Agency (EMA)/Committee for Medicinal Products for Human Use (CHMP). Guideline on Bioanalytical Method Validation, EMA/CHMP, London, United Kingdom.

15. Tırıs, G.; Tekkeli Kepekci S. E.; Önal, C.; et al. An UPLC method for the determination of sorafenib in human plasma by fluorimetric detection with pre-column derivatization and application to a pharmacokinetic study. *Rev. Roum. Chim.* 2022, *67,* 3, 217-224. https://doi.org/10.33224/rrch.2022.67.3.10

16. G. Heinzel, R. Woloszczak, S. Thomann, Version TOPFITT 2.0 Pharmacokinetic and Pharmacodynamic Data Analysis System for the PC. VCH Publishers: Gustav-Fisher-Verlag, Stuttgart, Germany, (1993).