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A Rabit Method for Quantification of Selexipag in Human Plasma Using High Performance Liquid Chromatography with Electron Spray Ionzationtendem Mass Spectrometry

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ABSTRACT

A simple, accurate and rabit method was developed using high performance liquid chromatography with electron spray ionzationtendem mass spectrometry (HPLC-ESI-MS) to quantify the concentration of selexipag in human plasma with K_2EDTA anticoagulant was developed and fully validated. Stable isotobically labelled compound selexipag D7 was used as an internal standard (ISTD). The sample extraction procedure utilized protein precipitation method. The chromatographic analysis was conducted on a Zorbax C18 XDB column (100x 4.6mm,i.d 3.5µm) within 5 min, using methanol with 5mM ammonium acetate (75:25%, v/v) was used as mobile phase at the flow rate of 0.7mL/min under an isocratic condition. The ionization was performed on electron spray ionization interference with positive mode by multiple reaction monitoring(MRM). The mass transions were 497.100→455.200 m/z for selexipag and 504.300→456.200 m/z for ISTD. Method validated as per USFDA guidelines and calibration curve was found to be linear in the range of 0.100-50.869 ng/mL.The results were within the acceptance limits. The extraction efficiency was 93.45% at the three quality control levels. The lower limit of detection (LLOQ) was found to be 0.104ng/mL.Stability studies demonstrated that selexipag was stable in plasma during Bench-Top (7hr at room temperatore), Auto-sampler (26hr 30 min at 4^{o}C), Freeze-Thaw (5cycles) and Long term analyte stability in plasma (41days at -20^{o}C).
Keywords: Selexipag;Human Plasma; Stability;Protein Precipitation; Validation.

INTRODUCTION

Selexipag belongs to the long-term treatment of pulmonary arterial hypertension (PAH) in adult patients (1). Chemically it is 2-[4-[(5,6-diphenylpyrazin-2-yl)-propan-2-ylamino]butoxy]-N-methylsulfonylacetamide (Fig:1a) and molecular formula $C_{26}H_{32}N_4O_4S$ with compound weight 496.6 g/mol. (2-3)

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The pharmacological act is choosy prostacyclin (IP, also called PGI2) receptor agonist. Initiation of the IP receptor brings vasodilation inside pulmonary circulation and inhibits the propagation of vascular smooth muscle cells, key factors in PAH pathogenesis. (4-5)

Suvorexant, approved in December 2015 in the United States Food and Drug Administration (USFDA) and also for use in January 2016, Canada. Developed by actelion pharmaceuticals ltd and nipponshinyaku in april 2008. Selexipag (Uptravi) is an oral route of administration. (6)

Drug literature review reveals that few analytical quantification methods have been reported for the selexipagin bulk, formulations, and biological matrices. Which includes UV spectrophotometric [7], high performance liquid chromatography [8] and ultra-high performance liquid chromatography tandem mass spectrometric detection (UPLC-MS/MS) [9-13].The present work designed to develop a simple,rugged, economic and validated RP-HPLC method for the determination of selexipag in human plasma with anti-coagulant K_2EDTA.

MATERIALS AND METHOD

Chemicals and Reagents

The pure standard of selexipag (purity 99.54% by hplc) and selexipag D7(Fig:1b) (purity 99.14% by hplc) as is basis were purchased from Vivan life sciences hydrabad, India. Emparta grade of ammonium acetate, LC-MS grade of methanol, deionized milli-Q-weter and acetonitrile purchased from Merck Specialties Private Limited, India. Glacial aecticacid purchased from Rankem Ltd., India, matrix: human plasma (K_2EDTA - Anticoagulant) acquired from a registered blood bank.

Instrument and Equipment

Quantitative analysis was performed on an Exion LC^{TM} chromatographic system (AB Sciex, USA). The detection of analyte and ISTD performed using ESI and triple quadrupole mass spectrometer API 6500. Data acquisition and processing were performed by using analyst software version 1.6.3 (AB Sciex) to control all parameters of LC and mass spectrometry. An agilentZorbax Eclipse XDB-C_{18} column of 100mm X 4.6mm: i.d and 3.5µm particle size with 300°A pore size was used. Micro weighing balance (MX5)- Mettler Toledo, an Ultrasonic bath sonicator, Deep Freezer (-20 ± 5°C) - Thermo Fisher Scientific, Refrigerator (2-8°C) - Thermo Fisher Scientific and Vortex- Spinix, Heidolph top were used in the study.

Chromatographicand MRM Condition

The chromatography separation of analytewas achieved by using zorbax eclipse XDB-C18 column (100×4.6 mm, 3.5µm) and the isocratic mobile phase consist of methanol: 5mM Ammonium actate (75:25%, v/v) was delivered with flow rate of 0.7mL/min without spilt. The mobile phase was degassed before use in an ultrasonicator bath for 5min. The column compartment (oven) and autosampler temperature were at 25^{o}C and 4^{o}C, respectively with an injection volume of 10 µL. the analysis run time was completed within 5min.The main working parameters of the mass spectrometer are given in table 1.

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Standard solution, Calibration Standard and Quality Control sample:

An accurately weighed amount of 5mg selexipag was transferred and dissolved into 5ml volumetric flask with Dimethyl Sulfoxide (DMSO). The solution is made upto the mark by using methanol diluent to get the concentration of 1 mg/mL.The working solutioncontaining selexipag of calibration standards were prepared by dilution of the standard stock solution with the diluent of60% methanol solution (v/v). Calibration standards in human plasma were prepared by spiking 2% of the corresponding working solutions with screened blank plasma matrix samples at the following concentrations: 0.100, 0.200, 0.400, 0.800, 5.128, 12.819, 21.365, 35.609 and 50.869 ng/mL for selexipag. The working solution for the quality control (QC) samples were prepared at the following concentrations: 0.100, 0.285, 15.845, 34.446 and 172.230 ng/mL for selexipag. ISTD working standard solution at the concentration of 500 ng/mL was prepared by diluting the standard stock solution (1 mg/mL) with the 60% methanol of diluent solution. The standard stock solution and working solution of calibration standard, quality control and ISTD samples were immediately stored at 2-8^{o}C.

Sample preparation

The sample preparation was performed by protein precipitation method. Exactly 0.100 mL of plasma sample was aliquoted and transferred into a 5mL tarsons RIA vial polypropylene tube and 0.050 mL of ISTD (500ng/mL) working concentration solution was added, except for standard blank, to which 0.050 mL of 60% methanol solution (v/v) was added and the mixture was vortexed for 30 sec. To this 0.5mL of 100% acetonitrile was added and vortexed for 5min. Centrifuged the allsamples for 10 min at 5000 rpm in 4ºC. Following centrifugation,the supernatant solution was transferred into auto sampler glass loading vials and injected 10 µL of the sample into the chromatographic system.

METHOD VALIDATION

Method validation was done as per the criteria of industrial guidance for bioanalytical method validation of USFDA[14].

System Suitability

System suitability was evaluated by analyzing6 repeated injections from same vial of standard aqueous mixture equivalent to an about middle concentration of the calibration curve of selexipag and working concentration of ISTD during the start of the method validation and at the start of the respective day. The area ratio and retention time (Analyte and ISTD) of system suitability has within the toleance limits of 5% CV.

Carryover Effect

Carryover effect was performed in order to remove the carryover from the previous injection to the next injection. Extracted blank, LLOQ and ULOQ samples were prepared from biological matrix of human plasma as mentioned above extraction process. These samples were injected in

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the sequnce of mobile phase, extracted blank (without analyte and ISTD), extracted LLOQ, extracted ULOQ and above extracted blank plasma samples during the start of the method validation. The area of interfering peaks at the RT of analyte has ≤ 20% of area of extracted LLOQ and at the RT of ISTD have ≤ 5% of area of extracted LLOQ.

Selectivity/ Specificity

The selectivity of the method was evaluated by analysing ten different lots of human plasma matrix which included two hemolyzed and two lipemic lots. From each lot, blank and LLOQ were processed using the above extraction method. For specificity, interference from analyte was established by processed minimum of six individual matrix lot with MQC concentration level without ISTD and interference from ISTD was established by processed minimum of six individual matrix lot with working concentration of ISTD without analyte. To examine the potential interferences of endogenous compounds at the LC peak region for selexipag and ISTD. The peak area of LLOQ for selexipag and ISTD at corresponding retention time in blank samples should not be more than 20% and 5% of the mean peak area of ISTD from passed CC's and QC's.

Sensitivity

Assessed the sensitivity in the terms of percentage accuracy and precision which was denoted by %CV. It was evaluated with the lower limit of quantification (LLOQ QC) 0.100ng/mL of quality control sample along with all precision and accuracy bathch. The tolerance limit of percentage accuracy within ±20 and %CV ≤20.

Calibration curve

Calibration curve was constructed by plotting the ratio of peak area of selexipag and selexipag D7against the nominal concentration of calibrators. The calibration curve were fitted by weighting factor $1/X^2$least square linear regression equation method (y=mX+c) which are distributed throughout the calibration curve range from 0.100to 50.869ng/ml of selexipag. The curve constructed by using balnk, zero and nine non-zero standards0.100, 0.200, 0.400, 0.800, 5.128, 12.819, 21.365, 35.609 and 50.869 ng/mL. The tolerance limit of calibration curve was a correlation coefficient (R^2) of 0.98 or greeter, and each back-calculated standard concentration have ±15% deviation from the nominal value with the exception of LLOQ, which was set at ±20%.

Precision and Accuracy

Precision and accuracy batch was calculated by analysing four batches. For P&A studies five concentration level of quality control samples were prepared as lower limit of quantification (LLOQ), lower quality qontrol (LQC), medium quality control (MQC), high quality control (HQC) and dilution integrated quality control (DIQC)equivalent to 0.100, 0.285, 15.845, 34.446 and 172.230 ng/mL respectively, with six replicates each. The intra-run and inter-run precision (% CV) for LOQ, MQC, HQC and DIQC should be ≤ 15% except for LLOQ, which was set at

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≤20% andthe intra-run and inter-run accuracy for LQC, MQC, HQC and DIQC should be within ±15% except for LLOQ, which was set at within ±20%.

Recovery

The percentageextraction efficency of selexipag from human plasma was calculated by comparing the mean peak response of six extracted low, medium and high (0.285, 15.845, and 34.446ng/mL) respectively, quality control samples to the mean peak response of six post-extracted low, medium and high quality control samples with the same concentrations.

Thepercentageextraction effiency of ISTD from human plasma was calculated by comparing the mean peak area of the prepared extracted ISTD to the mean peak area of post extracted ISTD at the concentration level intended for use. The % recovery of analyte and ISTD has to be less than 110%.

Matrix Factor

Matrix factor was evaluated at LQC and HQC level by using ten screened different lots of human plasma matrix which included one hemolyzed and one lipemic lots. To determine the matrix factor two sets of ten blank matrices were processed using the above extraction method. Post extraction samples were prepared by the standard of LQC and HQC containing internal standard were spiked into the extracted black matrices. In the same way, standard aqueous solution equal to LQC and HQC concentration containing internal standard was prepared using diluent and mobile phase as injected single batch. The acceptance criteria for IS normalised matrix effect was that the %CV should be less than 15 %.

Dilution Integrity

Dilution integrity was evaluted to ensure that samples could be diluted with screened blank matrix of human plasma without affecting the final concentration. Selexipag spiked human plasma samples were prepared at concentrations of 172.230 ng/mL,above the upper limit of the calibration range. These samples were further diluted with human pooled plasma five times dilution in six replicates and analysed with all P&A batch. The six replicates have a precision of ≤ 15%CV and accuracy of 100 ± 15%.

Ruggedness

The ruggedness of the method was assessed by the deliberate changes in the experimental state with a precision and accuracy batch. The batch was supervised using a similar chemistry type of column to another column manufacture (Phenomenex Luna C_{18}) and different analyst in the same laboratory.

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Run Size Evaluation

Evaluate the run size during method validation, which should include the number of samples to be analyzed under a run during actual study sample analysis. Establish run-size based on the chromatographic run time and analyte stability.

Reinjection Reproducibility

Reproducibility is the precision between two laboratories. It also represents the precision of the method under the same operating conditions over a short period. Re-injection reproducibility shall be evaluated by re-injecting anyone of the accepted P & A.

Stability experiments

The aim of determining the stability of selexipag in human plasma performed viz. bench-top stability, freeze-thaw stability, auto-injector stability, wet extract stability, Long-term analyte stability in plasma, stock and working solution stability studies were carried out by using six replicates of the lower and higher quality control samples.

The stability was calculated by comparing the found concentration to the nominal concentration values against the freshly prepared calibration standard and bracketed run acceptance quality control (LQC, MQC and HQC) samples.

Stock and working solution stability

To assess the standard stock solution stability of analyte and ISTD, stability samples were prepared and maintained at 2-8^{o}C for 16 days. The percentage bias calculated mean peak area of of the stability standard stock solution of analyte and ISTD against the comparable freshly prepared standard stock solution of analyte and ISTD, then injected six replicates of fresh and stability samples at LQC and HQC level.

Bench-top stability

To determine the stability of analyte in human plasma on the based-top condition, six replicates of stability quality control (LQC and HQC) samples were set separately at ambient temperature up to 7 hours then extracted and qualified.

Freeze-Thaw stability

Freeze Thaw stability of analyte was evaluated by six replicates of stability quality control (LQC and HQC) samples were frozen at -20 degree in the deep freezer. The frozen plasma samples containing the analyte thawed at room temperature for a minimum 1 hour followed by refrozen for minimum 12 hours. The stability quality control samples were exposed to 5FT cycles before being extracted and analysed.

Auto-sampler stability

To determine the stability of processed sample in autosampler condition, six replicates of stability quality control (LQC and HQC) samples were processed and left in the autosampler rack up to 26 hours 30 minutes at 4^{o}C then injected and quantified.

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Wet Extract stability

To determine the stability of wet extract, six replicates of stability quality control (LQC and HQC) samples were processed and stored at 2-8^{o}C refrigerator condition for 25 hours as wet extract form prior to loading into LC autosampler.

L**ong-term analyte stability in plasma**

To determine the long-term stability of analyte in plasma, six replicates of 3 set stability quality control (LQC, HQC and DIQC) samples were stored at -20^{o}Cin the deep freezer for 41 days. after completion of stability duration extracted and analyzed.

All stability experiments were stable if assay values were within the adequate tolerance of ±15% of accuracy and ≤15%CV of precision.

RESULTS AND DISCUSSION

The ionization techniques of positive and negative MRM mode was tried using Harvard syringe pump was carried out to obtain Q1 and Q3 ion mass spectra of analyte and ISTD with electron spray ionization probe source and the signal intensity was good and higher in the positive mode of ionization tuning. For selexipag and selexipag D7, the highly sensitive transitions were detected from precursor ion m/z 497.100 to product ion(Fig:2 a, b) m/z 455.200 and precursor ion m/z 504.300to product ion m/z 456.200(Fig:3 a, b), respectively.

Optimization of chromatographic condition to the proposed method required various trials using different mobile phases with modified compositions and stationary phases. The finest conditions were attained with isocratic elution using reversed phase zorbax eclipse XDB-C18 column (100×4.6 mm, 3.5µm). a mixture of methanol: 5mM ammonium actate (75:25%, v/v) was used as the mobile phase operated at a flow rate of 0.70 mL per minute. The peak achieved were well defined symmetric peak shape and good response at lower concentration with the retention time of 2.68 min for analyte and 2.64 min for ISTD mode was suitable for the detection within a reasonable time of analysis less than 5 min.

Protein precipitation method was used for sample preparation since relativity inexpensive technique, good extraction efficiency as well as simple procedure. methanol and acetonitrile precipitation solvents were tried, but hundred percent is acetonitrile was found to be most effective for extraction of both analyte and ISTD with minimal matrix effect and reproducible recovery. As a result of good response of selection in spiked LLOQ samples begins by the sample aliquote volume 100µL has been used. Thus, enhancing the sensitivity and accuracy of the LC-MS/MSanalysis.These data indicate that the developed method is highly specific and selective for the analysis of selexipag in human plasma samples.

System Suitability

The system suitability %CVof the retention time was found to be 0.41-1.55% forfor selexipagand 0.33-2.61% for selexipag D7. The %CV of the peak area ratio was found to be 2.33 to 4.10%. Prior to suitability few equilibration injections were given, and the results were found to be within the acceptance.

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Carryover Effect

The results indicated that no carryover was observed throughout this chromatographic method for both selexipag and selexipag D7. It does not affect the precision and accuracy of the individual run.

Selectivity/ Specficity

Selectivity of the technique was verified on ten blank human plasma samples obtained from different volunteers. The chromatographic method determined analyte of interest in the analysed matrices without interference from endogenous components(Fig:4 a,b,c,d). This matrices lots were further selected for preparation of calibration curve and quality control samples. The % accuracy of individual lot's LLOQ samples were within the acceptable range of ±20%. The selectivity and specificity experiments ensured null interference at the retention time of analyte and ISTD. Table 2.

Linearity

The linearity of the method was demonstrated peak area ratio of analyte to ISTD was linear with reliable reproducibility over the concentration range of 0.100 to 50.869ng/mL figure 4. At nine non-zero calibrator levels. The correlation coefficient R^2 for the calibration curve(Fig:5) ranged from 0.9984 - 0.9999 for selexipag. Table 3.

Sensitivity/ Precision and Accuracy

the Precision and accuracy statistical data for QC's are summarised in table 2. The intra-run and inter-run precision for each concentration level within the range of 1.65 to 8.65%CV and 7.36 to 5.86 %CV, respectively and the intra and inter run accuracy for each concentration level was within the range of 92.67 to 112.53% and 98.26-104.68% respectively. The lowest concentration with %CV less than 20% was taken as LLOQ and was found to be 0.104 ng/mL. The result was summarized in table 4.

Recovery

The relative recovery for LQC, MQC and HQC of selexipag were found to be found 91.32%, 96.32% and 97.25% respectively. The percentage mean global recovery of analyte was found to be 94.96% with adequate position of 3.36% CV and the ISTDpercentage mean recovery was found to be 93.45%. the result data shows that the simple protein precipitation extraction procedure efficiently extracts selexipag as well as selexipagD7 from human plasma. The results were summarized in Table 5.

Matrix effect

The post-extraction spiked method indicated that no significant effect of matrix ion was observed at the retention time of analyte and ISTD for QC levels (LQC and HQC). The %CV was found to

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be IS normalised matrix factor 3.92 and 6.19, correspondingly. The result of matrix effect as within the acceptable limit.

Dilution integrity

Dilution integrity of selexipag was performed up to five fold. The percentage nominal values was found within the acceptance limit of ±15% and the diluted samples mean precision was 1.65 to 7.44 % and accuracy was 95.93 to 102.34 %.

Ruggedness

The present method was shown good ruggedness when it was performed by using different analyst and column of different manufacture. The accuracy and precision result wasacceptable range of 95.77 to 102.54 % and 1.21-6.66 % CV respectively.

Stability

The stabilityo0f seelexipagwas assessed under different environment expected to be encountered during the analytical process and sample storage. The analyte passed all the stability parameter tests viz. stock solution stability (16 days at 2-8^{o}C), Auto-sampler (26h 30min at 4^{o}C), Bench-top (7h), wet extract (25h at room temperature), Freeze-Thaw (5 cycles) and deep freezer stability (41days at -20^{o}C). There was no significant decrease of the analyte concentration was observed. The summary of the stability parameters statistical data for selexipagpresented in the table 6.

CONCLUSION

Ahighly sensitive, selective, specific, accurate and precise LC-MS/MS method for the quantification of selexipag in human plasma was developed.The extraction procedure of analyte in biological matrix simple with reproducible recovery and less matrix effect.Proposed chromatographicmethod was rapid, allowing for sample preparation procedure and analysis of a large number of sample in a short period of time and comprehensive method validation was carried out. All results were within the range of acceptable limits as specified in USFDA guidelines (2018). Hence, the developed method can be applied to PK and TDM studies in humans with desired precision and accuracy.

AUTHORS CONTRIBUTIONS

Both author have contributed equally.

CONFLICT OF INTERESTS

Declared none.

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a)

b)

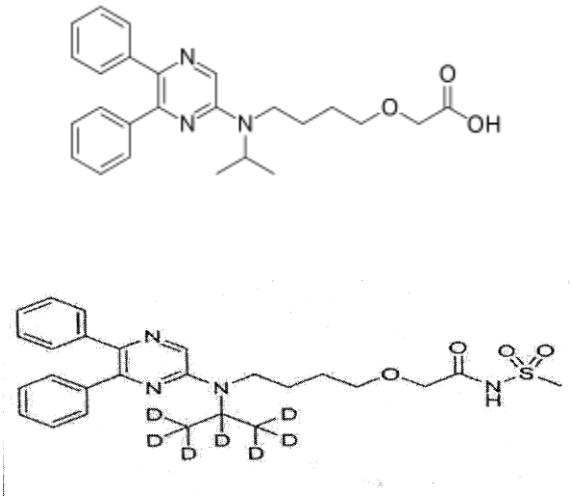
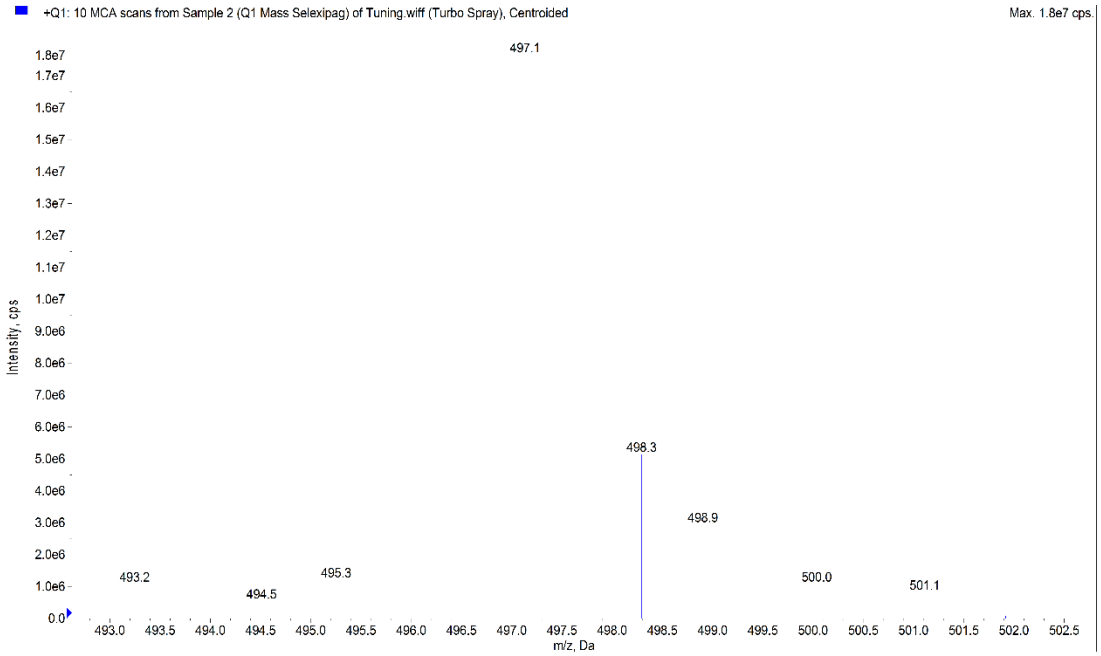


Fig. 1: Chemical structure of (a) Selexipag, (b) Selexipag D7 (ISTD)

(a)



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(b)

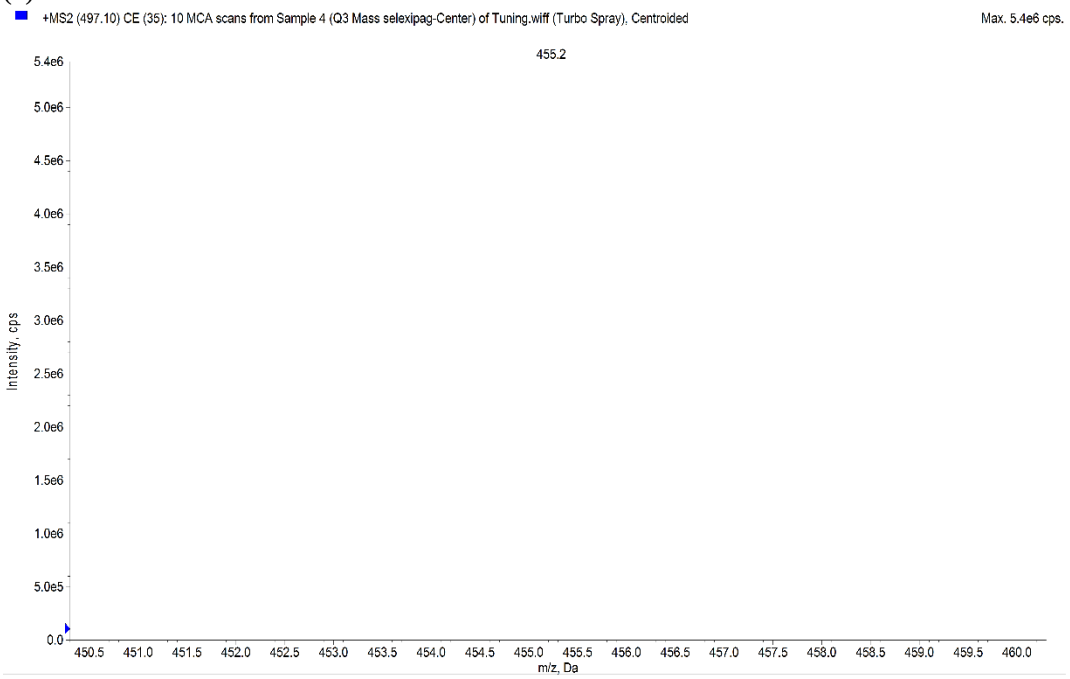
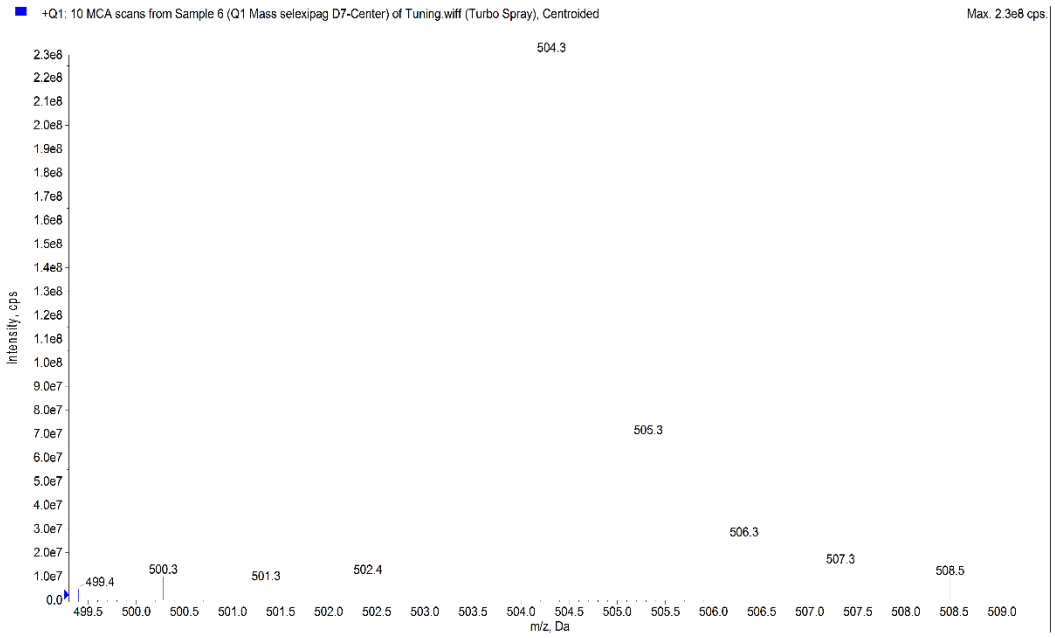


Fig. 2: Representative spectra for (a) SelexipagQ1, (b) SelexipagQ3.

(a)



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(b)

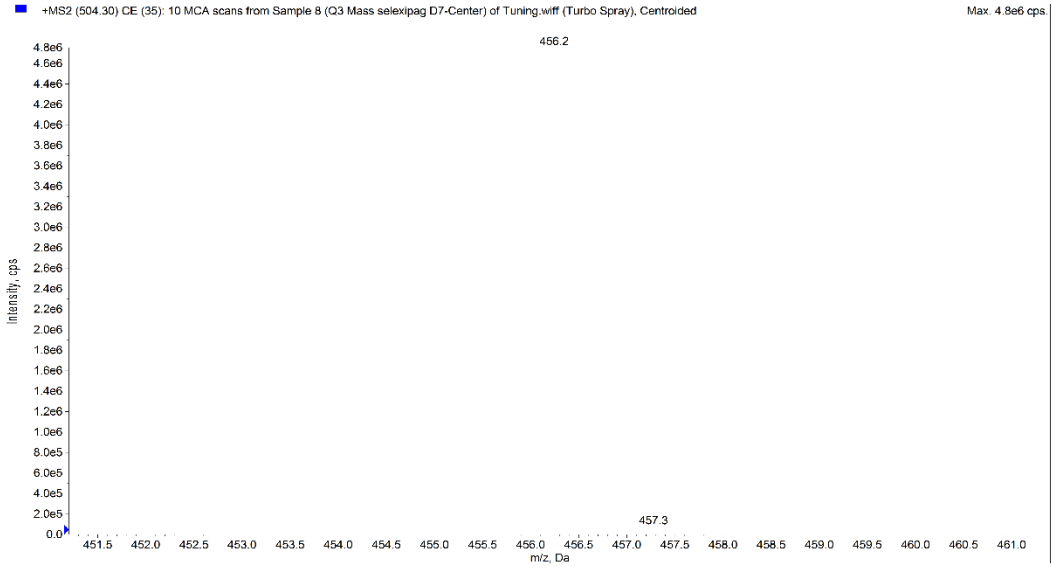
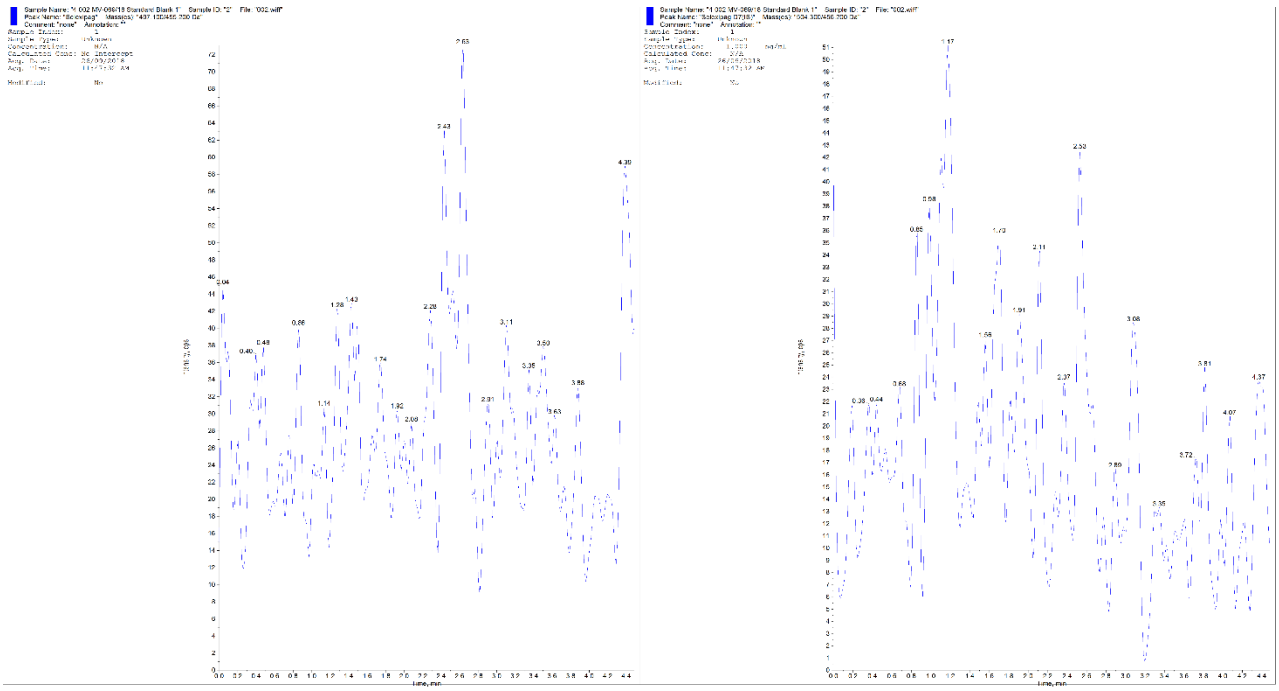


Fig. 3: Representative spectra for (a) SelexipagD7 Q1, (b) SelexipagD7 Q3.

(a)



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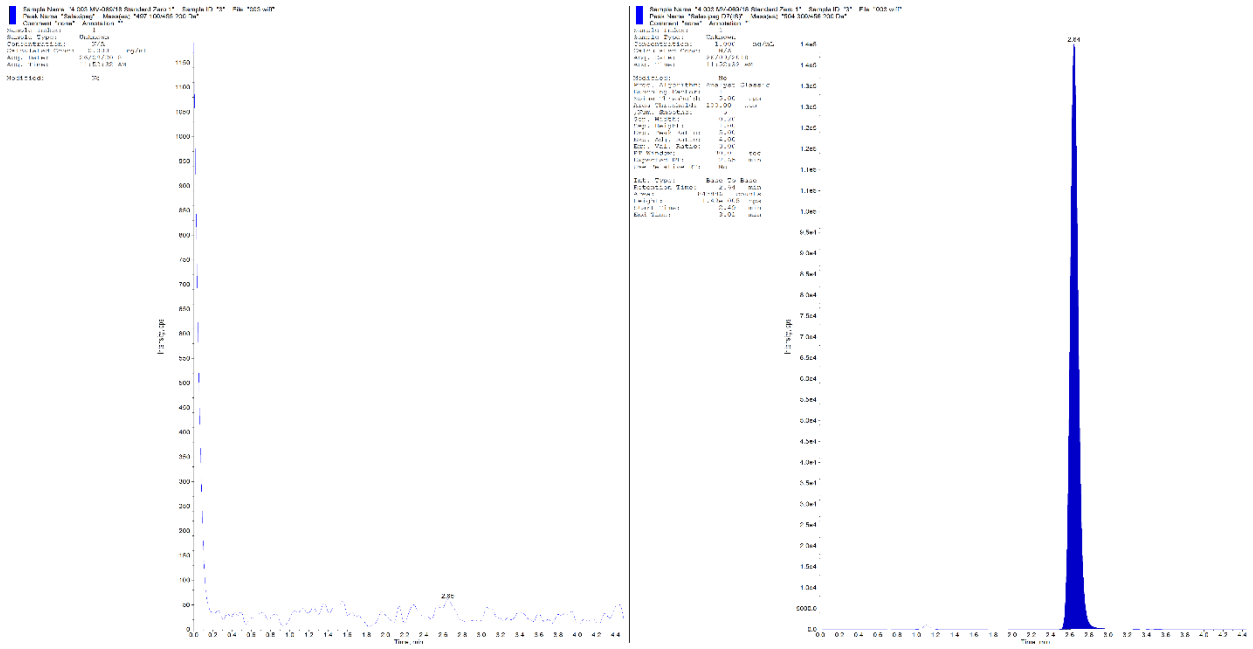
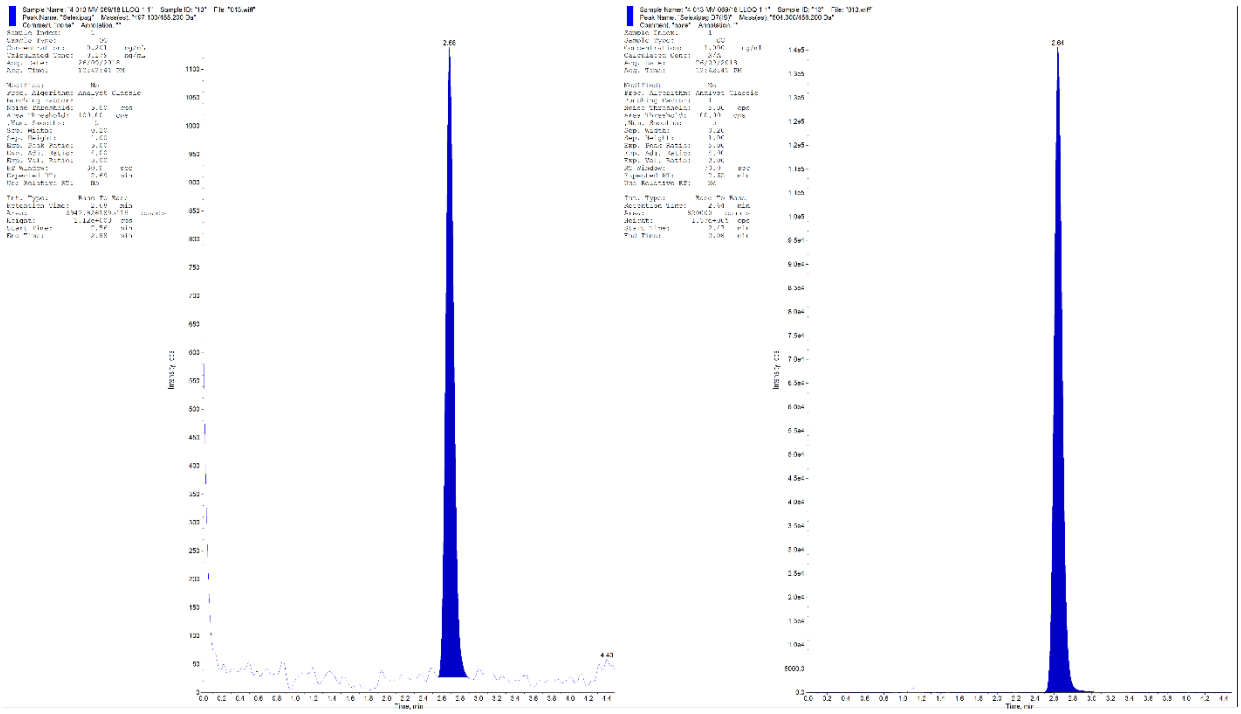
(b)



(c)



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(d)

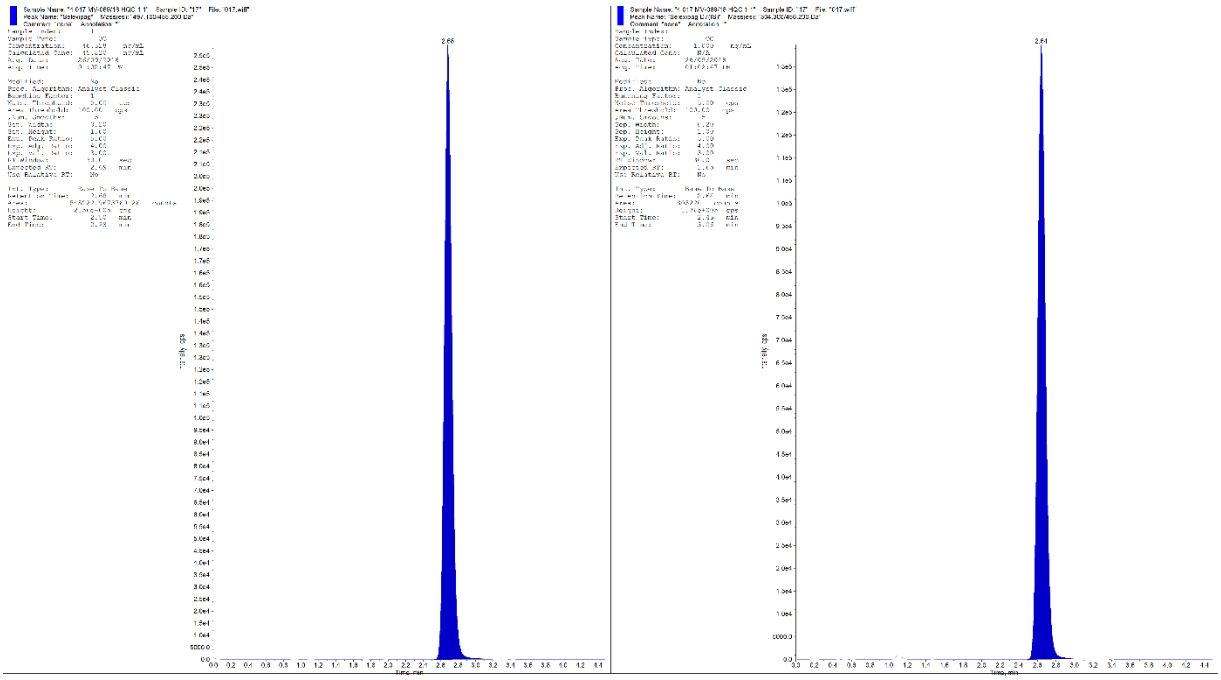

Fig. 4: Mass Chromatograms of (a) Blank plasma, (b) Blank plasma spiked with ISTD, (C) Blank plasma spiked with LLOQ, (d) Blank plasma spiked with ULOQ.

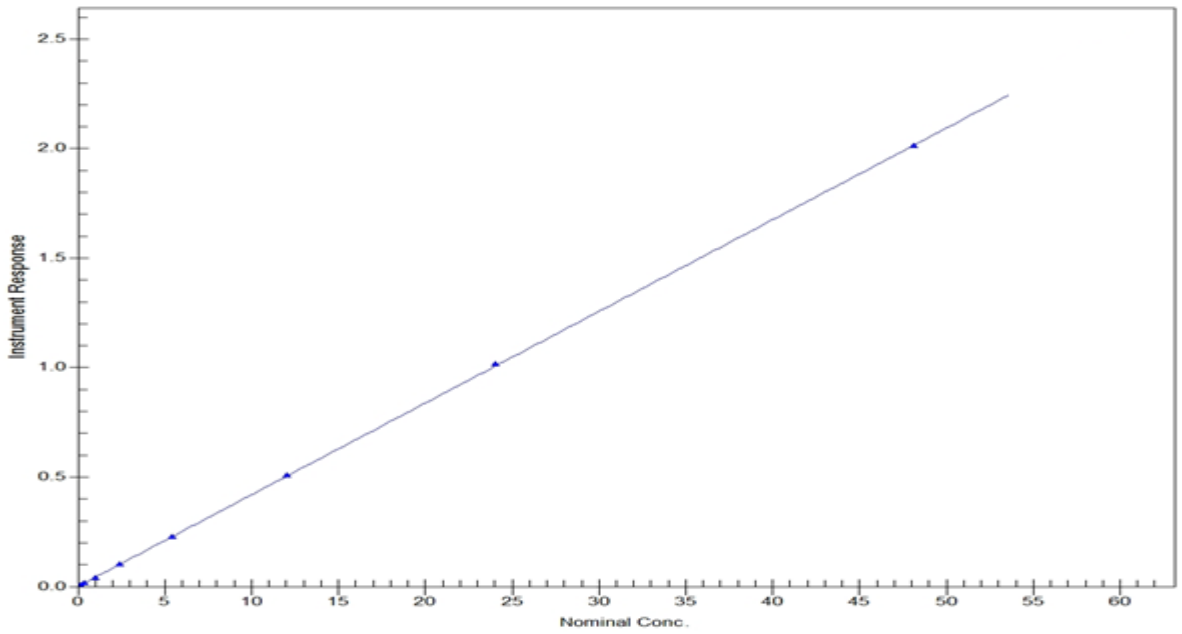

Fig. 5: Representative calibration curve for Selexipag in human K_2EDTA Plasma

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Table 1:Multiple Reaction Monitoring (MRM) conditions

Sl.no	Biological Matrix ID	Area response					
		Analyte Area (Blank)	Analyte Area (LLOQ)	% Inteference for Analyte	ISTD Area (Blank)	ISTD Area (LLOQ)	% Inteference for ISTD
1	Human Plasma Lot-1	0	4654	0	0	384587	0
2	Human Plasma Lot-2	0	4920	0	0	369954	0
3	Human Plasma Lot-3	0	4681	0	0	378201	0
4	Human Plasma Lot-4	0	4401	0	0	386355	0
5	Human Plasma Lot-5	0	4201	0	0	386123	0
6	Human Plasma Lot-6	0	5012	0	0	371147	0
7	Human Haemolysed Lot-1	0	5123	0	0	345720	0
8	Human Haemolysed Lot-2	0	4582	0	0	375243	0
9	Human Lipemic Lot-1	0	5147	0	0	363118	0
10	Human Lipemic Lot-2	0	5436	0	0	385552	0

Table 2: Selectivity-Interference from endogenous compound for Analyte and ISTD

Parameters	Selexipag	Selexipag D7
General Dependent		
Mass spectrometer	API 6500	
Tuning mode	Manual	
Ion source	Turbo Ion Spray (ESI)	
Ionization Mode	Positive Ionization	
Spray needle set point (X/Y)	5-May	
Compound Dependent		
Transition (m/z) Q1→Q3	497.100→455.200	504.300→456.200
Declustering Potential (V)	90	70
Entrance Potential (V)	10	10
Collision Energy (V)	30	30

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Collision Cell Exit Potential (V)	15	15
Source Dependent		
Curtain Gas (psi)	30	
Ion Spray Voltage (V)	5500	
Temperature (^{o}C)	500	
Gas Source 1 (psi)	40	
Gas Source 2 (psi)	45	
Collision gas (psi)	6	
Dwell Time Per Transition (msec)	200	

Table 3: Linearity

STD ID	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8	STD 9
Nominal concentration (ng/mL)	**0.100**	**0.200**	**0.400**	**0.800**	**5.128**	**12.819**	**21.365**	**35.609**	**50.869**
N*	4	4	4	4	4	4	4	4	4
Mean±SD	0.104	0.204	0.413	0.824	5.185	13.188	21.322	34.437	51.599
±SD	0.01	0.01	0.02	0.03	0.02	0.06	0.21	0.29	0.16
%CV	**8.64**	**5.79**	**3.96**	**3.37**	**0.38**	**0.43**	**0.99**	**0.85**	**0.30**
% Mean Accuracy	**104.47**	**101.96**	**103.27**	**103.01**	**101.12**	**102.88**	**99.80**	**96.71**	**101.44**

*Number of each concentration injections

Table 4: Precision and Accuracy for Intra run and Inter run

P& A	**LLOQ 0.100 ng/mL**	**LQC 0.285 ng/mL**	**MQC 15.845 ng/mL**	**HQC 34.446 ng/mL**	**DIQC 172.230 ng/mL**
N*	**6**	**6**	**6**	**6**	**6**
Intra-run Mean	0.107	0.304	17.009	32.659	165.212
Intra-run ±SD	0.00	0.02	0.27	1.05	2.72
Intra-run %CV	3.15	5.17	1.61	3.23	1.65
Intra-run % Accuracy	106.57	106.69	107.35	94.81	95.93
N*	**6**	**6**	**6**	**6**	**6**
Intra-run Mean	0.113	0.296	16.036	33.801	170.362

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P& A	LLOQ 0.100 ng/mL	LQC 0.285 ng/mL	MQC 15.845 ng/mL	HQC 34.446 ng/mL	DIQC 172.230 ng/mL
Intra-run ±SD	0.00	0.01	0.80	1.92	12.68
Intra-run %CV	3.91	3.91	5.01	5.69	7.44
Intra-run % Accuracy	112.53	103.89	101.21	98.13	98.92
N*	**6**	**6**	**6**	**6**	**6**
Intra-run Mean	0.093	0.312	15.219	34.232	168.885
Intra-run ±SD	0.00	0.01	0.29	2.96	7.45
Intra-run %CV	2.19	2.04	1.89	8.65	4.41
Intra-run % Accuracy	92.67	109.40	96.05	99.38	98.06
N*	**6**	**6**	**6**	**6**	**6**
Intra-run Mean	0.102	0.281	15.772	34.692	176.263
Intra-run ±SD	0.01	0.02	0.65	1.47	8.59
Intra-run %CV	5.44	5.89	4.10	4.25	4.87
Intra-run % Accuracy	102.45	98.74	99.54	100.71	102.34
N*	**24**	**24**	**24**	**24**	**24**
Inter-run Mean	**0.104**	**0.298**	**16.009**	**33.846**	**170.181**
Inter-run ±SD	**0.01**	**0.02**	**0.81**	**1.98**	**8.51**
Inter-run %CV	**7.36**	**5.04**	**5.06**	**5.86**	**5.00**
Inter-run % Accuracy	**103.56**	**104.68**	**101.04**	**98.26**	**98.81**

*Number of each concentration injections

Table 5: Recovery

QC ID	LQC		MQC		HQC		ISTD	
	Post Extracted Area	Extracted Area	Post Extracted Area	Extracted Area	Post Extracted area	Extracted Area	Post Extracted Area	Extracted Area
N*	6	6	6	6	6	6	6	6
Mean±SD	13579	12401	808934	779131	1657011	1611456	404486	377978
±SD	507	488	14148	15929	45581	49766	6641	6798
%CV	3.73	3.94	1.75	2.04	2.75	3.09	1.64	1.8
% Recovery	**91.32**		**96.32**		**97.25**		**93.45**	
%Global CV	**3.36**						**-**	
%Global recovery	**94.96**						**-**	

*Number of injections

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Table 6: Stability

Stability Experiment	QC ID	Nominal concentration (ng/mL)	Concentration found (ng/mL) (mean ± SD)*	Precision (% CV)	Accuracy (%)
Bench top Stability	LQC	0.285	0.278	8.05	97.58
	HQC	34.446	34.174	5.91	96.31
Auto sampler Stability	LQC	0.285	0.283	2.96	99.46
	HQC	34.446	31.981	5.03	92.84
Wet extract Stability	LQC	0.285	0.288	9.70	100.97
	HQC	34.446	31.825	5.45	92.39
Freeze thaw Stability	LQC	0.285	0.277	3.62	97.32
	HQC	34.446	32.406	5.41	94.08
Reinjection Reproducibility	LQC	0.285	0.296	5.59	103.77
	HQC	34.446	34.292	3.70	99.55
Long term plasma stability	LQC	0.285	0.286	6.07	100.24
	HQC	34.446	35.038	4.39	101.72
	DIQC	172.23	172.702	7.54	100.27

*Number of each concentration injections-6