Development and Validation of Bioanalytical Method for the Quantitative Estimation of Selexipag In Biological Matrices using LC-MS/ MS

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Abstract

The validated liquid-liquid extraction method was applied for estimation of Selexipag in human plasma with Selexipag-D6 as an internal standard (ISTD) by using LC-MS/MS. The chromatographic separation was achieved with Acetonitrile: 10mM Ammonium formate (pH-4.0) (80: 20, v/v) using the CORTECS C18 COLUMN (100 x 4.6 mm, 2.7 µ). The total analysis time was 10 min and flow rate was set to 0.5 ml/min. Detection was done by turboionspray (API) positive mode with unit resolution. Quantification was by MRM, where the acquired masses for Selexipag were $498.20 \rightarrow 344.20$ m/z and Selexipag-D6 was $503.70 \rightarrow 344.20$ m/z. The standard curve shows correlation coefficient (r^2) greater than 0.999 with a range of 10.00-25600.00 pg/ml using the linear regression model.

Keywords: Selexipag; Human plasma; LC-MS/MS; Bioanalysis

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a hemodynamic and pathophysiological condition affecting the pulmonary arterioles and characterized by progressive increases in pulmonary vascular resistance and pulmonary artery pressure, ultimately leading to right heart failure and premature death [1-3]. Recent therapeutic options have significantly improved the long-term outcome of patients with PAH, but PAH remains a disease with a poor prognosis [4-10].

Reduced expression of prostacyclin synthases in the lung and reduced levels of prostacyclin are key features of PAH. Prostacyclin is produced by endothelial cells from prostaglandin H2 (PGH2) by the enzyme prostacyclin synthase. Prostacyclin is a potent vasodilator and also has anti-proliferative, antithrombotic, and anti-inflammatory effects. As PAH is associated with vasoconstriction, proliferation, and thrombosis, there is a strong rationale for using prostacyclin treatment. Restoration of IP receptor signaling using prostacyclin receptor (IP receptor) agonists is an effective strategy in the treatment of the disease [11-14].

Selexipag is a novel, orally available, long acting (half-life of 6.2–13.5 h), highly selective IP receptor agonist that targets the prostacyclin pathway. Selexipag is a diphenylpyrazine derivative with a chemical structure (Figure 1) unrelated to prostacyclin and its analogues (e.g. it lacks the typical cyclopentane ring of prostacyclin analogues) [15-16].

Selexipag (Fig:1) is a selective non-prostanoid IP prostacyclin receptor agonist. The chemical name of selexipag is 2-{4-[(5,6-diphenylpyrazin-2-yl)(isopropyl)amino]butoxy}-*N*-(methylsulfonyl) acetamide. It has a molecular formula of $C_{26}H_{32}N_4O_4S$ of 496.62. Selexipag is a pale yellow crystalline powder that is practically insoluble in water. In the solid state selexipag is very stable, is not hygroscopic, and is not light sensitive [17-18].

Literature review shows that UV-VIS spectroscopy[19], HPLC[20] methods were reported for determination of selexipag in pharmaceutical formulations and none of the methods were reported for determination of selexipag in biological samples by LC-MS/MS using deuterated internal standard.

The present investigation reports a simple a simple, sensitive, precise LC-MS/MS method for the analysis of Selexipag in plasma based on the LLE with ethyl acetate. The developed method was validated as per FDA guidelines [21-23].

MATERIALS AND METHODS

Materials:

Chemical Resources

Selexipag (SP) and Selexipag-D6 (SPIS) (Fig:1.0) were procured from Hetero Pharmaceuticals, Hyderabad, India. Water (HPLC Grade), Ammonium acetate, (analytical grade) were purchased from Merck, Mumbai, India. Acetonitrile (HPLC Grade), Methanol (HPLC grade), Ethyl acetate (HPLC grade) were obtained from J.T. Baker, USA. Human plasma was procured from Clinim Blood Blank, Hyderabad. Milli Q water was taken from the in-house Milli-Q system.

Instrument Resources

An API 4000 HPLC-ESI-MS/MS system (Applied Biosystems), 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany), data acquisition and processing were accomplished using Analyst® Software 1.4.1.

Methods:

Chromatographic conditions

The chromatographic separation was achieved with 0.1% formic acid in combination with acetontrile (50:50 v/v, gave the best peak shape and low baseline noise was observed using the CORTECS C18 COLUMN (100 x 4.6 mm, 2.7 µ). The total analysis time was 10 min and flow rate was set to 0.5 ml/min. The temperature was set to 40°C for the column oven. The sample volume for the

injection into mass spectrometry was adjusted to 10 µl for better ionization and chromatography.

Detection
The pure drug of SP and SPIS were prepared in methanol (10.00 ng/mL) and injected with a flow rate of 5 µL/min into positive ion mode mass spectrometer for optimization of mass parameters like source temperature, IS, heater gas, nebulizer gas, curtain gas, CAD gas (all gas channels were purged with ultra high pure nitrogen gas), EP, DP, CE, FP and CXP were optimized. Analysis was performed using MRM positive ion mode with mass transitions of 498.20 → 344.20 m/z and Selexipag-D6 was 503.70 → 344.20 m/z. The mass fragmentation pattern of parent and product ions mass spectras were depicted in figure 2 and 3.

Standard calibration and quality control samples preparation
Standard stock solutions of SP (1.0mg/mL) and SPIS (1.0 mg/mL) were prepared in Methanol. The IS spiking solution (100.0pg/mL) was prepared in mobile phase solution (Acetonitrile: 10mM Ammonium formate (pH-4.0) (20: 80, v/v) from SPIS stock solution. Standard stock solutions and IS spiking solutions were stored in refrigerator conditions of 2–8°C until analysis. Standard stock solutions of SP (1.0 mg/mL) were added to drug-free screened human plasma to obtain concentration levels of 10, 50, 100, 200, 400, 800, 1600, 3200, 6400, 12800 and 25600 pg/mL for analytical standards, and 10 (LLOQ), 30 (LQC), 30720 (MQC) and 40960 pg/mL (HQC) for quality control (QC) standards, and stored in the freezer at 30°C until analysis. The aqueous standards were prepared in a mobile phase solution (Acetonitrile: 10mM Ammonium formate (pH-4.0) (20: 80, v/v) and stored in the refrigerator at 2–8°C until analysis.

Sample extraction
The LLE method was used to isolate SP and SPIS from human plasma. For this purpose, 50µL of SPIS (100 pg/mL) and 100µL of plasma sample were added to the labelled polypropylene tubes and vortex briefly for about 5 min. Thereafter 3mL of extraction solvent of ethyl acetate was added and vortex for about 10 min. Next, the samples were centrifuged at 4000 rpm for approximately 5 min at ambient temperature. From each, a supernatant sample was transferred into labelled polypropylene tubes and evaporated to a dryness of 40°C briefly, and then reconstituted with a mobile phase solution (Acetonitrile: 10mM Ammonium formate (pH-4.0) (20: 80, v/v), and the sample was transferred into autosampler vials and injected into the LC-MS/MS for study.

Method validation
The developed method was validated over a linear concentration range of 10.0–25600.0 pg/ml. The validation parameters include selectivity and specificity, LOQ, Linearity, precision and accuracy, matrix effect, recovery, stability (freeze–thaw, auto sampler, bench top, long term) was evaluated under validation section [221-23].

Selectivity and Specificity
Ten lots of blank plasma samples were analyzed out of which six lots free from interference were selected for assessing the selectivity and specificity. The endogenous/potential interfering peak areas for blank samples must be less than 20% of the LLOQ peak area of SP retention time and less than 5% for SPIS retention time.

Limit of Quantification (LOQ)
Six LLOQ standards were prepared in screened plasma lot along with IS (100.00 pg/ml) and signal to noise ratio (S/N) was calculated using analyst software.

Linearity
Calibration standards were prepared to obtain linearity range of 10, 50, 100, 200, 400, 800, 1600, 3200, 6400, 12800, 25600 pg/mL and assayed in five replicates on five different days.

Precision & Accuracy
One set of calibration standards and one set contains four different concentrations of quality control standards of 10 pg/mL (LLOQ), 30 pg/mL (LQC), 30720 pg/mL (MQC) and 40960 pg/mL (HQC) concentrations were prepared in screened plasma and analyzed each quality control (QC) standards in six replicates on the same day (Intra day) and five different days (Inter day).

Matrix Effect
Six extracted blank plasma samples in three replicates were spiked with the un-extracted concentration of mid QC (30720.00 pg/ml) and compared with un-extracted standards of the same concentration.

Recovery
The recovery of samples was performed by protein precipitation method. The extraction recovery was determined in sextuplicate by comparing the extracted QC standards with un-extracted QC standards at three different concentrations of low (30.00 pg/ml), medium (30720.00 pg/ml), high (40960.00 pg/ml).

Stability studies

Bench top Stability (Room Temperature Stability, 30 h)
Six replicates of spiked low and high concentrations (Bench top stability samples) were set aside at ambient temperature up to 30 h. Samples were processed and compared with newly prepared low and high concentrations (comparison samples).

**Freeze and thaw stability (after 3rd cycle at -70°C)**
Six replicates of low and high concentrations (FT stability samples) were frozen at -30°C and subjected to three freeze-thaw cycles of 24, 36 and 48 h (-70°C to room temperature) and compared with newly prepared low and high concentrations (comparison samples).

Autosampler stability (2-8°C, 48 h)
Six replicates of low and high concentrations (AS stability samples) were stored in auto-sampler up to 48 h at 2-8°C. Stability samples were compared with newly prepared low and high concentrations (comparison samples).

Long-term Stability (-70°C, 90 Days)
After completion of the stability period stored at -70 °C (90 days) six replicates of low and high concentrations (LT stability samples) were compared with newly prepared low and high concentrations (comparison samples).
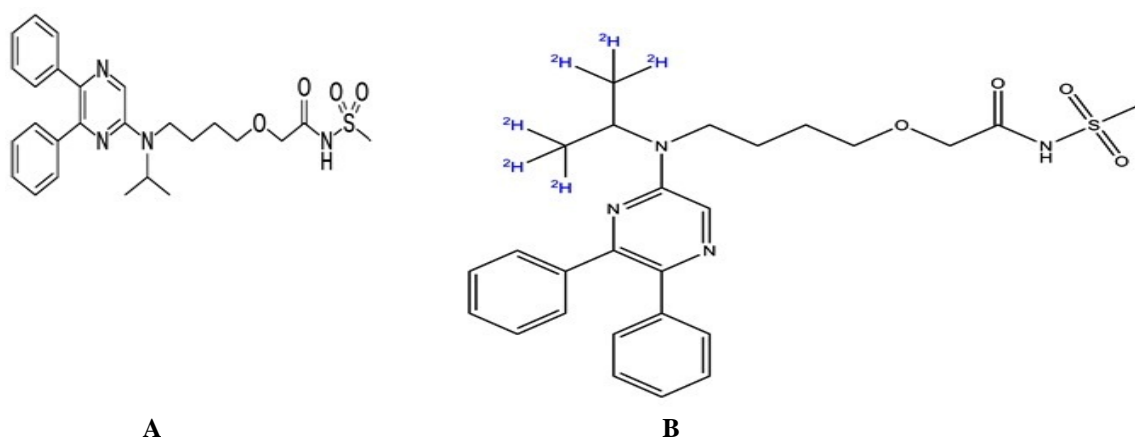
A   B

Fig.1: Chemical Structure A) Selexipag (SP) B) Selexipag-D6 (SPIS)

Table. 1 - Calibration curve details

Spiked plasma Concentration (pg/ml)	Concentration measured (pg/ml) (Mean±S.D)	%CV (n=5)	%Accuracy
10	10.18± 0.11	1.09	101.80
50	50.34± 0.38	0.75	100.68
100	100.30 ± 0.45	0.45	100.30
200	200.36± 0.15	0.07	100.18
400	400.48 ±0.28	0.07	100.12
800	800.40 ±0.27	0.03	100.05
1600	1600.31 ± 7.07	0.44	100.02
3200	3182.56 ± 40.49	1.27	99.46
6400	6386.24 ± 31.22	0.49	99.78
12800	12781.99 ± 108.60	0.85	99.86
25600	25598.20 ± 17.94	0.07	99.99

Table.2: Precision and accuracy (Analysis with spiked samples at three different concentrations)

Spiked Plasma Concentration (pg/ml)	Within-run (Intra-day)			Between-run (Inter-Day)		
	Concentration measured (n=6;pg/ml;mean±S.D)	%CV	%Accuracy	Concentration measured (n=6;pg/ml;Mean±S.D)	%CV	%Accuracy
10.00	10.50 ± 0.25	2.36	104.99	10.14 ± 0.57	5.65	99.10
30.00	30.56 ± 0.23	0.77	101.86	30.68 ± 0.26	0.84	102.27
30720.00	30716.46 ± 5.55	0.02	99.99	30750.58 ± 18.77	0.06	100.10
40960.00	40918.40 ± 32.73	0.08	99.90	40945.22 ± 8.61	0.02	99.96

Table.3 - Stability studies of Selexipag in plasma

Spiked Plasma concentration (pg/ml)	Room temperature Stability		Processed sample Stability		Long term stability		Freeze and thaw stability	
	30h		48h		90 days		Cycle (48h)	
	Concentration measured (n=6;pg/ml; Mean±S.D)	%CV (n=6)	Concentration measured (n=6;pg/ml; Mean±S.D)	%CV (n=6)	Concentration measured (n=6;pg/ml; Mean±S.D)	%CV (n=6)	Concentration measured (n=6;pg/ml; Mean±S.D)	%CV (n=6)
30.00	29.44±1.14	3.86	29.89±0.82	2.75	29.42±2.05	6.97	30.74±0.65	2.10
40960.00	40581.90±182.96	0.45	41017.57±35.35	0.09	40801.70±925.91	2.27	40059.41±547.80	1.37

RESULTS AND DISCUSSION

Method development

On the way to develop a simple and easy applicable method for determination of SP in human plasma, HPLC-MS/MS was selected as the method of choice. During method development process chromatographic (mobile phase composition, column, flow rate, injection volume, sample volume), mass spectrometric, sample extraction and internal standard parameters were optimized in logical and sequential manner to achieve the best results.

Separation of the SP was performed with different branded RP-HPLC C$_{18}$ columns. Initial separation was performed with isocratic elution of 10mM ammonium formate and acetonitrile was selected as a mobile phase in varying combinations were tried, but a low response was observed. A mobile phase consisting of (Acetonitrile: 10mM

Ammonium formate (20: 80, v/v) gave the best response, but poor peak shape was observed.

After a series of trials a mobile phase consisting of 10mM Ammonium formate(pH:4.0) in combination with Acetonitrile and acetonitrile in varying combinations were tried. Using a mobile phase containing 10mM Ammonium formate(pH:4.0) in combination with acetontrile (20:80 v/v), gave the best signal along with a marked improvement in the peak shape and low baseline noise was observed using the CORTECS C18 COLUMN (100 x 4.6 mm, 2.7 µ) mm analytical column with a flow rate of 0.5 ml/min and reduced runtime to 10 min. The column oven temperature was kept at a constant temperature of about 40°C and temperature of auto sampler was maintained at 4°C. Injection volume of 10 µl sample was adjusted for better ionization and chromatography. For selection of internal standard; Propranolol - Metoprolol - Atenolol - Bisoprolol were tried with optimized mobile phase and column conditions. Finally selexipag-D6 (SPIS) was selected as IS (internal standard) due to its compatibility with analyte chromatographic conditions. SP and SPIS were eluted at 5.10±0.05 and 5.25±0.05 min, with a total run time of 10 min for each sample.

Different procedures like PPT (Protein precipitation), SPE (solid phase extraction) and LLE (liquid-liquid extraction) methods were optimized. Out of all, it was observed that the LLE (liquid-liquid extraction) was suitable due to simple extraction, high recovery and the less ion suppression effect on drug and internal standard.

Electro spray ionization (ESI) provided a maximum response over atmospheric pressure chemical ionization (APCI) mode, and was chosen for this method. The instrument was optimized to obtain sensitivity and signal stability during infusion of the analyte in the continuous flow of mobile phase to electrospray ion source operated at a flow rate of 20 µl/min. Brigatinib gave more response in positive ion mode as compare to the negative ion mode.

To get high intense productions source dependent parameters were optimized like nebulizer gas flow 30 psi, CAD gas and curtain gas flow 25 psi, ion spray voltage 5500 V, and temperature 500°C. The compound dependent parameters such as the declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE), cell exit potential (CXP) were optimized during tuning for Selexipag and Selexipag-D6, respectively. The collision activated dissociation (CAD) gas was set at 5 psi using nitrogen gas. Quadrupole-1 and quadrupole-3 were both maintained at a unit resolution and dwell time was set at 180 ms for Selexipag and Selexipag-D6.

The predominant peaks in the primary ESI spectra of SP and SPIS correspond to the MH^+ ions at m/z 498.20 and 503.70 respectively. Productions of SP and SPIS scanned in quadrupole-3 after a collision with nitrogen in quadrupole-2 had a m/z of 344.20 for both respectively. The parent and productions mass spectrums of SP and SPIS were shown in Figure 2 & 3.
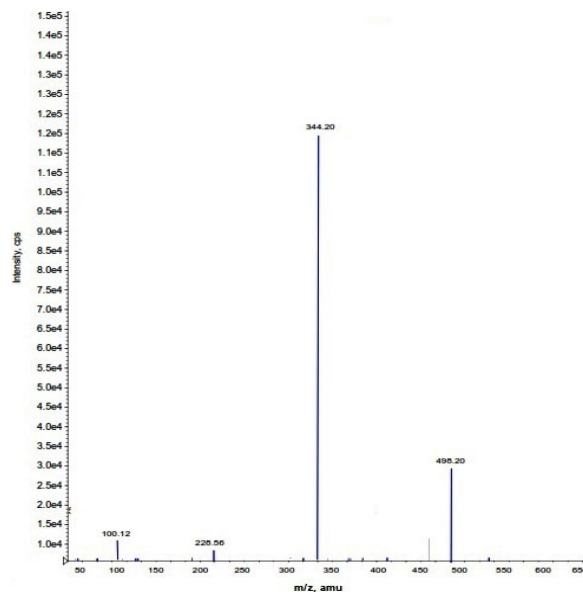

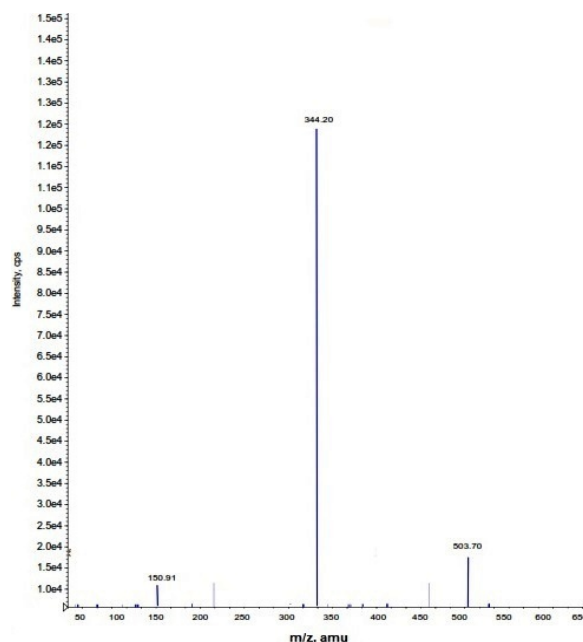**Figure.2. Mass scan spectrum of selexipag**
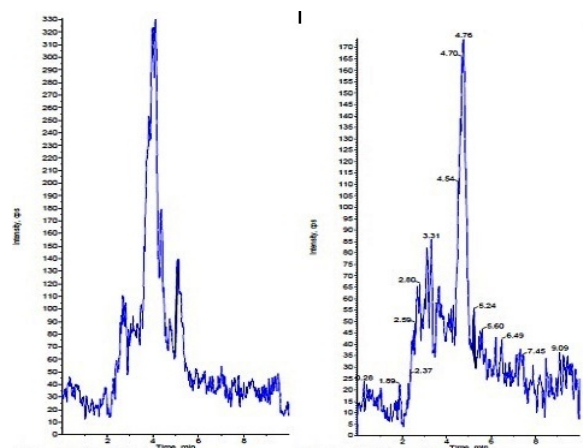

Figure.3. Mass scan spectrum of selexipag-D6

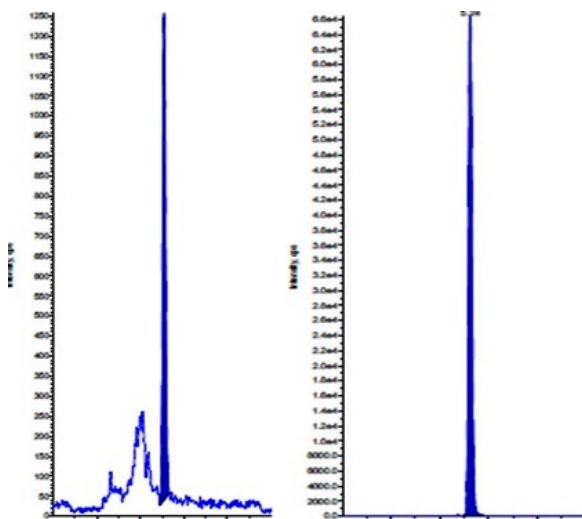**Figure.4: Blank plasma chromatogram of interference free SP and SPIS**

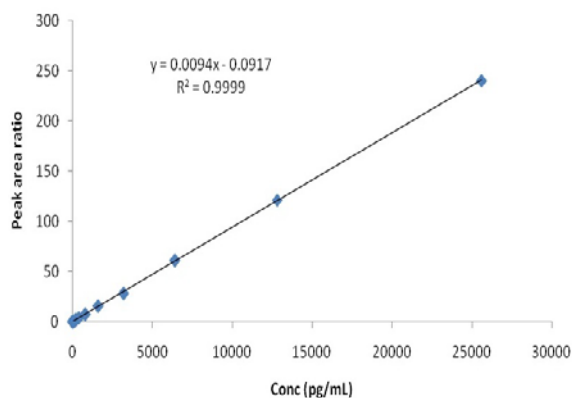**Figure.5: Chromatogram of LOQ sample (SP and SPIS)**

$y = 0.0094x - 0.0917$
$R^2 = 0.9999$

Figure.6: Calibration curve of SP

Method validation
Selectivity and Specificity, Lower limit of quantification (LLOQ)
No significant response was observed at retention times of SP and SPIS in blank plasma as compared to LLOQ and blank with IS samples. The limit of quantification for this method was proven as the lowest concentration of the calibration curve which was proven as 10.0 pg/ml. Represent chromatograms were shown in Figure 4 and 5.

Linearity
Linearity was plotted as a peak area ratio (SP peak area / SPIS peak area) on the y-axis against SP concentration (pg/ml) on the x-axis. Calibration curves were found to be consistently accurate and precise for SP over a linearity range of 10 to 25600 pg/ml. The correlation coefficient was greater than 0.99980 for SP. The %CV was less than 15% and mean %accuracy was ranged between 98.13 – 99.98%. Results were presented in Fig:6 and Table:1.

Precision & Accuracy
Intra and inter batch %accuracy for SP was ranged between 97.40-101.62 and 93.30 to 107.01. %CV is 10.04 to 4.60 and 4.31% - 7.85%. Results are presented in Table 2.

Recovery
The mean %recovery for LQC, MQC, HQC samples of SP were 96.25%, 97.72% and 95.25% respectively. The overall mean %recovery and %CV of SP across QC levels is 96.24% and 1.02%. For the SPIS (internal standard) the mean % recovery and %CV is 100.02% and 1.77%.

Matrix Effect
No significant matrix effect found in different sources of rat plasma tested for SP, SPIS. The %CV was found to be 9.70.

Stability (freeze–thaw, auto sampler, bench top, long term)
Quantification of the BT in plasma subjected to three freeze–thaw cycles (−30°C to room temperature), autosampler (processed), room temperature (Benchtop), long-term stability details were shown in Table 3.

CONCLUSION
The method described in this manuscript has been developed and validated over the concentration range of 10.0–25600.0 pg/ml in human plasma. The intra and inter-batch precision (%CV) was less than 15.0% and %accuracy ranged from 99.90 to 104.99 %. The overall % Recovery for SP, SPIS was greater than 90%. The selectivity, sensitivity, precision and accuracy obtained with this method make it suitable for the purpose of the present study. In conclusion, the method used in the present study is easy and fast to perform; it is also characterized with an adequate accuracy, precision, selectivity and stability. The simplicity of the method, and using rapid liquid-liquid extraction with run time of 10.0 min per sample, make it an attractive procedure in high-throughput bioanalysis of Brigatinib.

Conflict Of Interest:
Authors declare that, there is no conflict of interest.

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