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A NEW LC-MS/MS METHOD FOR DETERMINATION OF CABOZANTINIB AND NIVOLUMAB IN RAT PLASMA

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ABSTRACT
The present paper describes a simple, accurate, precise bio analytical method based on liquid-liquid extraction. It has been developed and validated for quantification of Cabozantinib and Nivolumab in rat plasma. Simple isocratic chromatographic condition and mass spectrometric detection has been demonstrated using this method with a QTRAP-5500 system. Linear range 2-40 mg/ml was identified during validation for Cabozantinib, whereas, Nivolumab linear range is 0.5-10 mg/ml. In addition, we have also reported the intraday precision and interday precision % RSD values for Cabozantinib and Nivolumab. Finally we have identified the overall recovery for Cabozantinib is 98.5% and Nivolumab is 99.2%.

1. INTRODUCTION
Generally Cabozantinibis is a medication that can be used for various treatments such as medullary thyroid cancer [I] renal cell carcinoma [II]. In addition, it is also worked as inhibitor [III] of the tyrosine kinesis [IV] c-Met [V-VI] VEGFR2 [VII], AXL [VIII], RET [IX]. Similarly, Nivolumab is also used for treatement of cancer [XI] and metastatic melanoma [XII]. On the other hand, if the cancer does not have a mutation in BRAF as a second-line [XIII] treatment following treatment with Ipilimumab and if the cancer has a mutation in BRAF, with a BRAF inhibitor [XIV] as a second-line treatment for squamous non-small cell lung cancer [XV] and as a second-line treatment for renal cell carcinoma [XVI]. Especially, it should not give to pregnant women in order to protect the baby health. However, they provide side effects include severe immune-related inflammation of the lungs, colon, liver, kidneys, thyroid and there are effects on skin, central nervous system [XVII], the heart and the digestive system [XVIII]. It is a human IgG4 [XIX] anti-PD-1 [XX] monoclonal antibody [XXI-XXIII]. It was discovered at Medarex [XIV], developed by Medarex and Ono pharmaceutical, and brought to market by Bristol-Myers Squibb [XXV]. To the best of our knowledge quantification of Cabozantinib and Nivolumab was reported by

HPLC [XXVI], however, no reports are available using LC-MS. Thus, here in, we would like to report the method development and validation of Cabozantinib and Nivolumab with more accurate by LC-MS method.

2. Experimental

Cabozantinib, Cabozantinib-D6 and Nivolumab, Nivolumab-D6 were obtained from Zyduscadila, Ahmedabad. Acetonitrile (LC-Grade) was purchased from J.T. Baker Inc. Orthophosphoric acid (HPLC grade) was purchased from Merck (Mumbai, India). Rat plasma was obtained from Bharat biotech, Hyderabad.

2.1 Instrumentation

An HPLC system (Waters Alliance e2695 model) connected with mass spectrometer QTRAP 5500 triple quadrupole instrument (Sciex) was used. Data processing was performed with Empower 2.0 software.

2.2 Detection

Initially the detection was examined with mass spectrometer. The mass spectrometer was operated in the multiple reaction monitoring (MRM) modes. Sample introduction and ionization were electrospray ionization were electrospray ionization in the positive ion mode. Sources dependent parameters optimized were as follows: nebulizer gas flow 30psi; curtain gas flow 25psi; ion spray voltage 2000v; temperature (TEM) 375°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 as 40, 35, 10, 12, 8eV for Cabozantinib and Nivolumab. The collision activated dissociation (CAD) gas was set at 4 psi using nitrogen gas. Quadrupole 1 and quadrupole 3 were both maintained at a unit resolution and dwell time was set at 300 ms for Cabozantinib and Nivolumab. The mass transitions were selected as m/z 505.8 → 395.4 for Cabozantinib and 662.8 → 550.2 for Nivolumab. The data acquisition was ascertained by Empower-2.0 software.

2.3 Chromatography

Waters symmetry C18 (4.6 mm x 150 mm, 3.5µm) was selected as the analytical column. Mobile phase composition was 0.1% Orthophosphoric acid: Acetonitrile (50:50 v/v). Source flow rate was 300µl/min without split with injection volume of 10µl. Cabozantinib and Nivolumab were eluted at 5.16±0.2 min and 6.08±0.2 min, with a total run time of 8.0 min for each sample.

2.4 Calibration curve and quality control samples

The stock solutions of Cabozantinib and Nivolumab were prepared with acetonitrile at free base concentration of 20 mg/ml and 5 mg/ml. Primary dilutions and working standard solutions were prepared from stock solutions using acetonitrile: water (50:50 v/v) solvent mixture. These working standard solutions were used to prepare the calibration curve and quality control samples. Blank rat plasma was screened prior to spiking to ensure it was free of endogenous interference at retention times of Cabozantinib and Nivolumab. Eight point standard curve and four quality control samples were prepared by spiking the blank plasma with an appropriate amount of Cabozantinib and Nivolumab. Calibration samples were made with various concentrations like 2.0, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 40.0 µg/ml of Cabozantinib, similarly, 0.5, 1.25, 2.5, 3.75, 5.0, 6.25, 7.5, 10.0 µg/ml concentration Nivolumab samples were also made. Quality control samples were also prepared with various concentrations of 1.0, 10.0, 20.0, 30.0µg/ml of Cabozantinib and 0.25, 2.5, 5.0, 7.5µg/ml of Nivolumab.

2.5 Sample Preparation

Internal standard (500µl) was added into the mixture of acetonitrile (500µl) and plasma sample (500µl). The precipitation mixture was mixed properly with the help of vortex cyclo mixture machine at 400 rpm for 20 mins. Collect the supernatant solution in HPLC vial and inject into the chromatogram.

2.6 Selectivity

Selectivity was performed by analyzing the rat plasma samples from six different rats to test for interference at the retention time of analytes.

2.7 Matrix effect

Matrix effect for Cabozantinib and Nivolumab with internal standards was evaluated by comparing the peak area ratio in the post extracted plasma sample from 6 different drug-free blank plasma samples and neat reconstitution samples. Experiments were performed at MQC levels in triplicate with six different plasma lots with the acceptable precision (% CV) of ≤15%.

2.8 Precision and accuracy

It was determined by replicate analysis of quality control samples (n = 6) at a lower limit of quantification (LLOQ), low quality control (LQC), medium quality control (MQC), high quality control (HQC) levels. The %CV should be less than 15% and accuracy should be within 15% except LLOQ where it should be within 20%.

2.9 Recovery

The extraction efficiencies of Cabozantinib and Nivolumab were determined by analysis of six replicates at each quality control concentration. The percentage recovery was evaluated by comparing the peak areas of extracted standards to the peak areas non extracted standards.

2.10 Stability

Stock solution stability was performed by comparing the area response of analyte and internal standard in the stability sample, with the area response of sample prepared from fresh stock solution. Stability studies in plasma were performed at the LQC and HQC concentration levels using six replicates at each level. Analyte was considered stable if the change is less than 15% as per US FDA guidelines [XXVII]. The stability of spiked rat plasma samples stored at room temperature (bench top stability) was evaluated for 24h. The stability of spiked rat plasma samples stored at 2-8°C in autosampler (autosampler stability) was evaluated for 24h. The autosampler sample stability was evaluated by comparing the extract plasma samples that were injected immediately, with the samples that were reinjected after storing in the autosampler at 2-8°C for 24h. The reinjection reproducibility was evaluated by comparing the extracted plasma samples that were injected immediately, with the samples that were re-injected after storing in the autosampler at 2-8°C for 24h. The freeze-thaw stability was conducted by comparing the stability samples that had been frozen at -30°C and thawed three times, with freshly spiked quality control samples. Six aliquots each of LQC and HQC concentration levels were used for the freeze-thaw stability evaluation. For long-term stability evaluation the concentrations obtained after 24h were compared with initial concentration.

3. Results and discussion

3.1 Method development

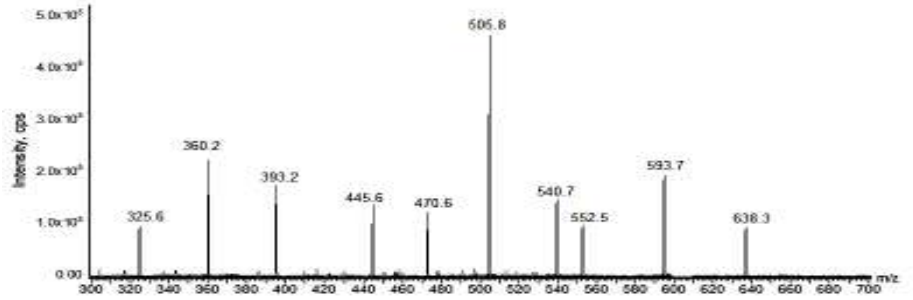
During method development, different options were evaluated to optimize mass spectrometry detection parameters, chromatography and sample extraction.

3.1.1 Mass spectrometry detection parameters optimization

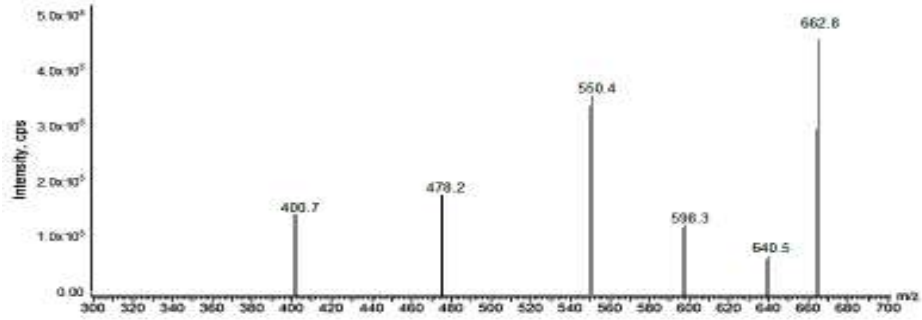
Electrospray ionization (ESI) provided a maximum response over atmospheric pressure chemical ionization (APCI) mode, and was chosen for this method. The instrument was

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 both polarities at a flow rate of 10µl/min. Cabozantinib and Nivolumab gave more response in positive ion mode as compare to the negative ion mode. The predominant peaks in the primary ESI spectra of Cabozantinib and Nivolumab correspond to the MH+ ions at m/z 505.8 and 602.3 respectively (Fig. 1A and B). Product ions Cabozantinib-D and Nivolumab-D6 scanned in quadrupole 3 after a collision with nitrogen in quadrupole 2 had an m/z of 498.6 and 586.4 respectively (Fig.1 C and D).

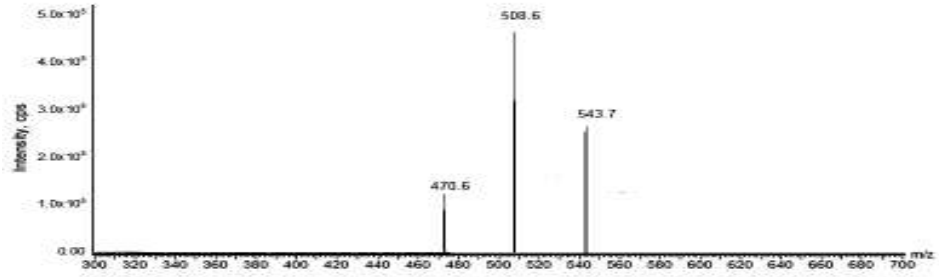
A



B



C



D

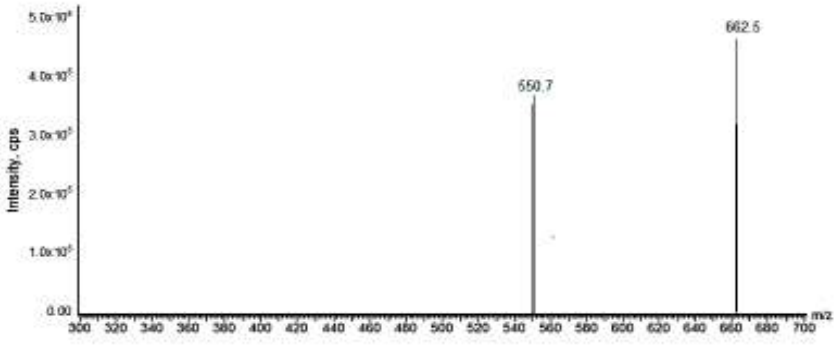


Figure 1 Mass spectra (A) Cabozantinib, (B) Nivolumab, (C) Cabozantinib-D6, (D) Nivolumab-D6

3.1.2 Chromatography optimization

Initially, a mobile phase consisting of water and acetonitrile in varying combination was tried, but a low response was observed. The mobile phase containing acetic acid: acetonitrile (20:80 v/v) having peak tailing and plate count is not within the limit. Mobile phase of 0.1% ortho phosphoric acid in water and acetonitrile with varying combinations was tried. The best signal along with a marked improvement in the peak shape was observed for Cabozantinib and Nivolumab using mobile phase containing 0.1% ortho phosphoric acid in water in combination with acetonitrile (50:50 v/v). Symmetry C18 (150mmx4.6mm, 3.5µ) column was used; it gave satisfactory peak shapes for both Cabozantinib and Nivolumab. Flow rate 1.0ml/min. Drugs and internal standards were eluted in shorter time at 8min.

3.1.3 Extraction optimization

Prior to load the sample for LC injection, the co-extracted proteins should be removed from the prepared solution. For this purpose, initially we tested with different extraction procedure like protein precipitation (PPT), Liquid-liquid extraction (LLE) and solid phase extraction (SPE). We found ion suppression effect in protein precipitation method for drug and internal standard. Further, we tried with SPE and LLE. Out of all, we observed LLE is suitable for extraction of the drug and internal standard. We tried with several organic solvents (ethyl acetate, acetonitrile, chloroform, n-hexane, dichloromethane and methyl tertiary butyl ether) individually as well with combination in LLE to extract analyte from the plasma sample. In our case acetonitrile is good extraction solvent. Several compounds were investigated to find a suitable internal standard, and finally Cobazontinib-D6 and Nivolumab-D6 was found to be the most appropriate internal standard for the present purpose. There was no significant effect of IS on analyte recovery, sensitivity or ion suppression. High recovery and selectivity was observed in the liquid-liquid extraction method. These optimized detection parameters, chromatographic conditions and extraction procedure resulted in reduced analysis time with accurate and precise detection of Cabozantinib and Nivolumab in rat plasma.

3.2 Method validation

A thorough and complete method validation of Cabozantinib and Nivolumab in rat plasma was done following US FDS guidelines. The method was validated for selectivity, sensitivity, matrix effect, linearity, precision and accuracy, recovery, reinjection reproducibility and stability.

3.2.1 Selectivity and sensitivity

Representative chromatograms obtained from blank plasma and plasma spiked with a lower limit of quantification (LOQ) sample is shown in Fig 2 and 3 for Cabozantinib and Nivolumab. The mean % interference observed at the retention time of analytes between six different lots of rat plasma, including hemolyzed and lipedemic plasma containing K_2EDTA

as an anti-coagulant was 0.00% and 0.00% for Cabozantinib and Nivolumab respectively, which was within acceptance criteria. Six replicates of extracted samples at the LLOQ level in one of the plasma sample having least inference at the retention time of Cabozantinib and Nivolumab were prepared and analyzed. The % CV of the area ratios of these six replicates of samples was 1.1% for Cabozantinib and 1.5% for Nivolumab.

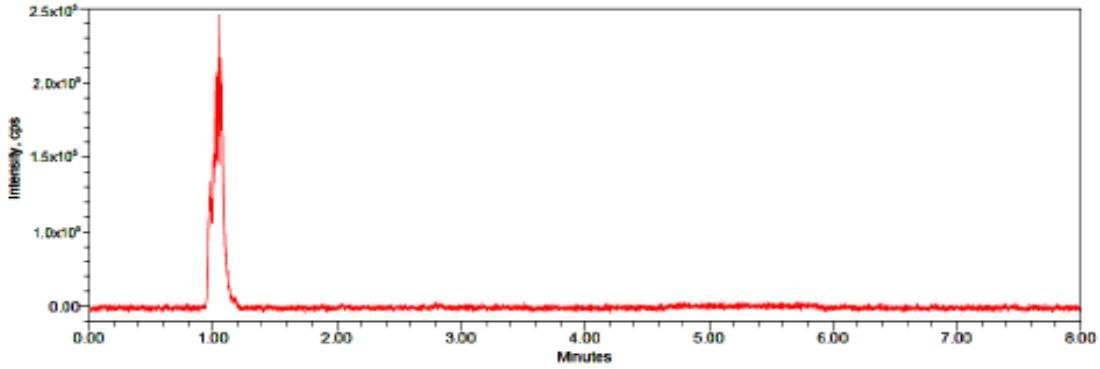


Figure 2 Blank plasma chromatogram for Cabozantinib and Nivolumab in rat plasma

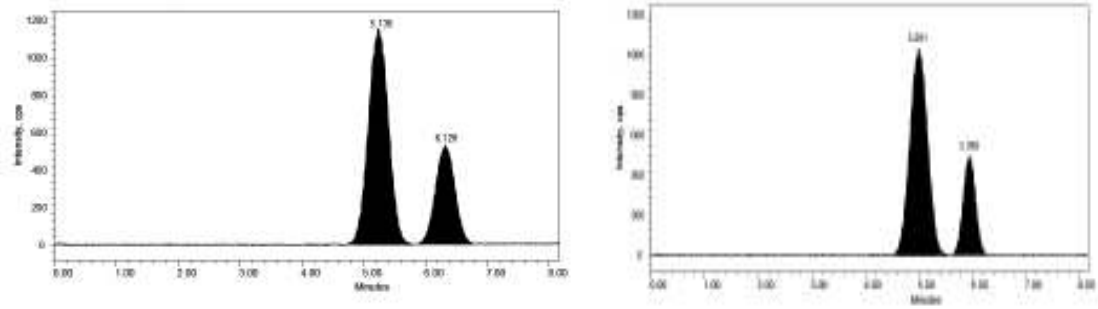


Figure 3 LLOQ chromatogram for Cabozantinib, Nivolumab and Cabpzantinib-D6, Nivolumab-D6 in rat plasma

3.2.2 Matrix effect

The % CV of ion suppression/enhancement in the signal was found to be 1.0% at MQC level for Cabozantinib and Nivolumab indicating that the matrix effect on the ionization of analyte is within the acceptable range under these conditions.

3.2.3 Linearity

The peak area ratios of calibration standards were proportional to the concentration of Cabozantinib and Nivolumab in each assay over the nominal concentration range of 2-40 ng/ml and 0.5-10 ng/ml. The calibration curves appeared linear and were well described by least squares linear regression lines in Fig 4&5. The correlation coefficient was ≥ 0.9991 for Cabozantinib and Nivolumab.

Table 1 Linearity data for Cabozantinib

Linearity	Cabozantinib Conc. (ng/ml)	Cabozantinib Peak response	IS Peak response	Area ratio
Linearity-1	2	0.201	2.011	0.1001
Linearity-2	5	0.515	2.036	0.2528
Linearity-3	10	1.022	2.021	0.5054
Linearity-4	15	1.536	2.044	0.7515
Linearity-5	20	2.046	2.074	0.9863
Linearity-6	25	2.531	2.018	1.2542
Lineaity-7	30	3.068	2.051	1.4959

Linearity-8	40	4.029	2.066	1.9501
Slope	0.049765			
Intercept	0.001250			
CC	0.999			

Table 2 Linearity data for Nivolumab

Linearity	Nivolumab Conc. (ng/ml)	Nivolumab Peak response	IS peak response	Area ratio
Linearity-1	0.50	0.104	2.011	0.052
Linearity-2	1.25	0.253	2.036	0.124
Linearity-3	2.50	0.509	2.021	0.252
Linearity-4	3.75	0.752	2.044	0.368
Linearity-5	5.00	1.058	2.074	0.510
Linearity-6	6.25	1.312	2.018	0.650
Linearity-7	7.50	1.526	2.051	0.744
Linearity-8	10.00	2.114	2.066	1.023
Slope	0.100641			
Intercept	0.001119			
CC	0.999			

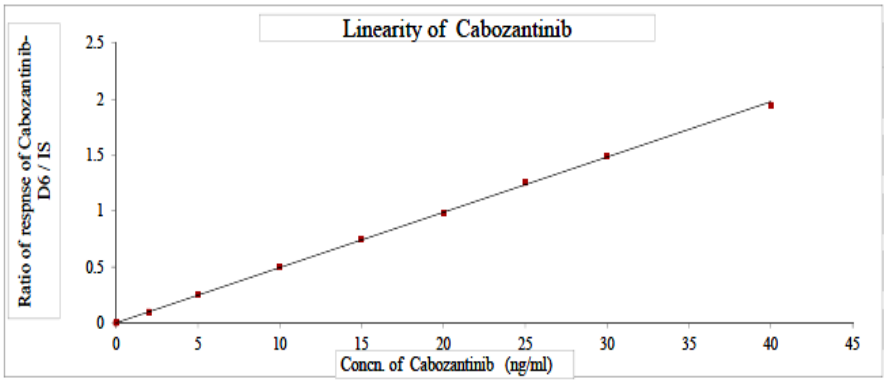


Figure 4 Calibration plot for Concentration v/s Area ratio of Cabozantinib

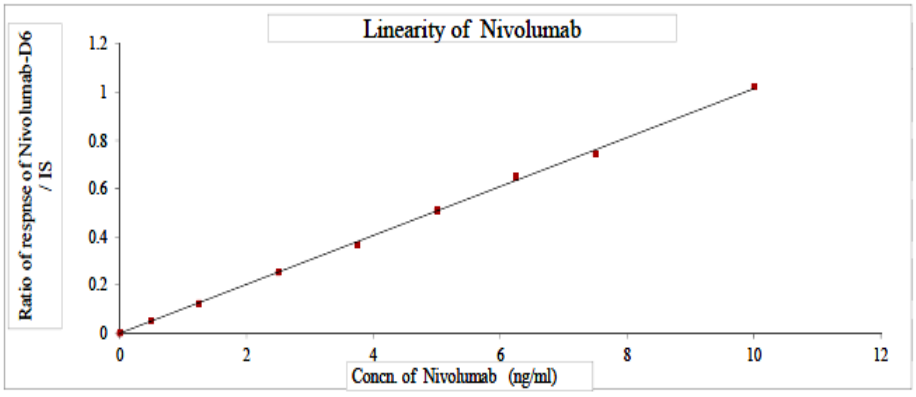


Figure 5 Calibration plot for Concentration v/s Area ratio of Nivolumab

3.2.3 Precision and accuracy

The inter-run and accuracy were determined by pooling all individual assay results of replicate (n=6) quality control over five separate batch runs analyzed on four different days.

The inter-run precision (% CV) was <5% and inter-run accuracy was in between 85 and 115 for Cabozantinib and Nivolumab. All these data presented in table 3and 4 indicate that the method is precise and accurate.

Table-3 Within-run and between-run precision and accuracy for Cabozantinib

Nominal Conc. (ng/ml)	Within run			Between run		
	Mean (ng/ml)	Precision (%CV)	Accuracy	Mean (ng/ml)	Precision (%CV)	Accuracy
1.0	0.99	0.85	98.5	1.01	1.12	97.8
10	10.01	1.02	99.8	10.0	1.15	98.3
20	19.98	1.34	100.2	20.1	0.98	99.8
30	29.99	0.98	100.1	30.1	0.59	100.1

Table-4 Within-run and between-run precision and accuracy for Nivolumab

Nominal Conc. (ng/ml)	Within run			Between run		
	Mean (ng/ml)	Precision (%CV)	Accuracy	Mean (ng/ml)	Precision (%CV)	Accuracy
0.25	0.24	0.69	99.3	0.25	0.98	99.8
2.5	2.51	1.01	99.9	2.49	0.75	100.1
5	5.01	0.98	100.1	5.02	0.69	100.2
7.5	7.49	1.13	99.8	7.50	1.16	99.5

3.2.5. **Recovery**

Six aqueous (sample spiked reconstitution-solution) at low , medium and high quality control concentration levels for Cabozantinib and Nivolumab were prepared for recovery determination , and the areas obtained for extracted samples of the same concentration levels from a precision and accuracy batch run on the same day. The mean recovery for Cabozantinib and Nivolumab was 98.2% with a precision of 1.2%. This indicates that the extraction efficiency for Cabozantinib and Nivolumab as well as Cabozantinib-D6 and Nivolumab-D was consistent and reproducible.

3.2.6 **Reinjection Reproducibility**

Reinjection reproducibility exercise was performed to check whether the instrument performance remains unchanged after hardware deactivation due to any instrument failure during real subject sample analysis. The change was less than 2.0 at LQC and HQC concentration levels hence batch can be reinjected in the case of instrument failure during real subject sample analysis. Furthermore , sample were prepared to be reinjected after 24h , which shows % change less than 2.0% at LQC and HQC concentration levels; hence batch can be reinjected after 24 h in the case of instrument failure during real subject sample analysis

3.2.7. **Stabilities**

Stock solution stability was performed to check stability of Cabozantinib and Nivolumab in stock solutions prepared in diluent and stored at 2-8° C in a refrigerator. The freshly prepared stock solutions were compared with stock solutions prepared before 24 hours. The % change for Cabozantinib and Nivolumab was 1.21% and 0.89% respectively which indicates that stock solutions were stable at least for 24 hours. Bench top and autosampler stability for Cabozantinib and Nivolumab was investigated at LQC and HQC levels.

Cabozantinib and Nivolumab were stable in plasma for at least 24 h at room temperature, and 24h in an auto sampler at 20°C. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples spiked with Cabozantinib and Nivolumab at LQC and HQC levels

did not affect their stability. The long-term stability results also indicated that Cabozantinib and Nivolumab were stable in a matrix up to 24 hours at a storage temperature of -30°C. The results obtained from all these stability studies are tabulated in Table 5and 6.

Table-5 Stability of the Cabozantinib

Stability experiments		Spiked Plasma concentration (n=6, ng/ml)	Concentration measured (n=6, ng/ml)	%CV (n=6)
Bench Top Stability	LQC	10	9.99	1.0
	HQC	30	30.12	0.9
Autosampler Stability	LQC	10	10.01	1.5
	HQC	30	29.99	1.2
Long term Stability	LQC	10	10.11	0.8
	HQC	30	30.05	1.3
Freeze-thaw Stability	LQC	10	10.08	1.1
	HQC	30	30.02	0.7

Table-6 Stability of the Nivolumab

Stability experiments		Spiked Plasma concentration (n=6, ng/ml)	Concentration measured (n=6, ng/ml)	%CV (n=6)
Bench Top Stability	LQC	2.5	2.48	0.6
	HQC	7.5	7.51	1.1
Autosampler Stability	LQC	2.5	2.50	0.8
	HQC	7.5	7.52	1.2
Long term Stability	LQC	2.5	2.49	0.5
	HQC	7.5	7.48	1.2
Freeze-thaw Stability	LQC	2.5	2.49	0.9
	HQC	7.5	7.52	1.1

3.3. **Application**

The validated method has been successfully to quantify Cabozantinib and Nivolumab concentration in 6 group of rats, under fasting conditions after administrations of 50 mg tablet containing Cabozantinib and Nivolumab as an oral dose. Drug sample was injected into rat body collected samples at different time intervals like 1, 2, 6, 8, 12, 18 and 24hrs. After that samples are prepared as per test method injected into chromatographic system recorded the values. The phrmacoknitics parameters evaluated were Cmax (maximum observed drug concentration during the study), AUC0-12 (area under the plasma concentration –time curve measured 6.5h , using the trapezoidal rule)tmax(time to observed maximum drug concentration),Kel (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 t1/2(terminal half-life as determined by the quotient 0.693/Kel, Table 7).

The Test/reference ratios for Cmax , AUC0-12 , and AUC were 80.56, 90.12 respectively, and they were within the acceptance range of 80%-125%demonstrating the bioeqvalence of the two formulations of Cabozantinib and Nivolumab. The mean concentration versus time

profile of Cabozantinib and Nivolumab in rat plasma as test and reference is show in figure 6,7.

Table-7 Mean pharmacokinetic parameters of Cabozantinib and Nivolumab

Pharmacokinetic Parameters	Cabozantinib	Nivolumab
AUC_{0-t}(ng h/ml)	2065.17	501.02
C_{max}(ng/ml)	2412.33	524.36
$AUC_{0-\infty}$ (ng h/ml)	2015.87	500.36
K_{el}	0.6498	0.3548
$t_{1/2}$	24	24
t_{max} (h)	12	18

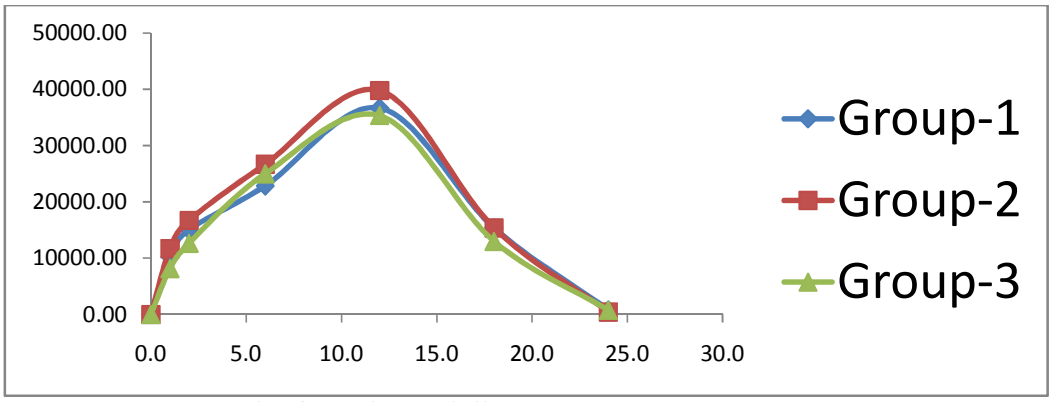


Figure 6 Recovery plot for Cabozantinib

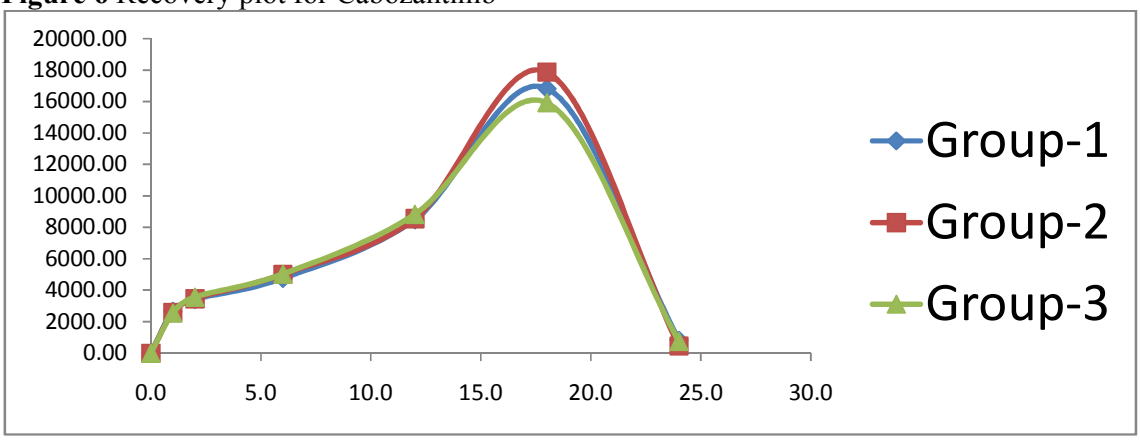


Figure 7 Recovery Plot for Nivolumab

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