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RESEARCH ARTICLE

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF CABOZANTINIB & NIVOLUMAB IN COMBINED PHARMACEUTICAL DOSAGE FORMS BY RP-HPLC

Tadikonda Rama Rao and Jadhav Bharath
Department of Pharmaceutical Analysis, CMR College of Pharmacy, Hyderabad, Telangana, India

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Abstract

A simple, accurate and precise HPLC method for simultaneous determination of Cabozantinib and Nivolumab in pure and tablet dosage form has been developed. HPLC of Waters (Model: Alliance 2695) with Phenomenax Luna C18(4.6 mm × 250 mm, 5 µm) column was used for chromatographic separation. Mobile phase consists of Methanol: Water (65:35% v/v) and flow rate adjusted was 1 ml/min. Wavelength selected for detection was 220 nm and injection volume was 10 µl. The method has been validated for linearity, accuracy, and precision. The linearity of Cabozantinib and Nivolumab were in the range of 30 µg/ml respectively. The developed HPLC method offers several advantages such as rapidity, usage of simple mobile phase and easy sample preparation steps. Further, improved sensitivity makes it specific and reliable for its intended use. Hence, this method can be applied for the analysis of pure drug and pharmaceutical dosage forms. From the present study it can be concluded that the proposed method is simple, sensitive, precise, specific, accurate and reproducible. Results of validation parameters demonstrated that the analytical procedure is suitable for its intended purpose and meets the criteria defined in ICH.

Introduction:-

In the modern pharmaceutical industry, high-performance liquid chromatography (HPLC) is the major and integral analytical tool applied in all stages of drug discovery, development, and production. It is ideal for the analysis of many drugs in both dosage forms and biological fluids due to its simplicity, high specificity and good sensitivity. High Performance Liquid Chromatography (HPLC) is a technique that has arisen from the application to liquid chromatography, the use of an instrumentation that was originally developed for gas chromatography. High Pressure Liquid Chromatography was developed in the mid-1970 and was improved with the development of column packing material and the additional convenience of on-line detectors. The various components of HPLC are pumps (solvent delivery system), mixing unit, gradient controller and solvent degasser, injector (manual or automatic), guard column, analytical columns, detectors, recorders and/or integrators. Recent models are equipped with computers and software for data acquisition and processing.

The mobile phase in HPLC refers to the solvent being continuously applied to the column or stationary phase at a flow rate of 1-5 cm^3/min. The mobile phase acts as a carrier for the sample solution. The chemical interactions of the

Corresponding Author:- Tadikonda Rama Rao
Address:- Professor & Principal CMR College of Pharmacy, Kandlakoya Village, Medchal Road, Hyderabad - 501401, Telangana, India.

mobile phase and sample with the column determine the degree of migration and separation of components contained in the sample. The mobile phase can be altered to manipulate the interactions of the sample and the stationary phase.[1-28]

Drug Profile:

Table 1:- Drug profile of Cabozantinib

Drug	Cabozantinib
Synonym	Cabozantinib
Category	Antineoplastic Agents
IUPAC	N'1-{4-[(6,7-dimethoxyquinolin-4- yl)oxy]phenyl}- N1-(4fluorophenyl) cyclopropane-1,1-dicarboxamide
Molecular formula	$C_{28}H_{24}FN_3O_5$
Molecular weight	501.514 gm/mole
pK_a	13.46
Log p	4.01

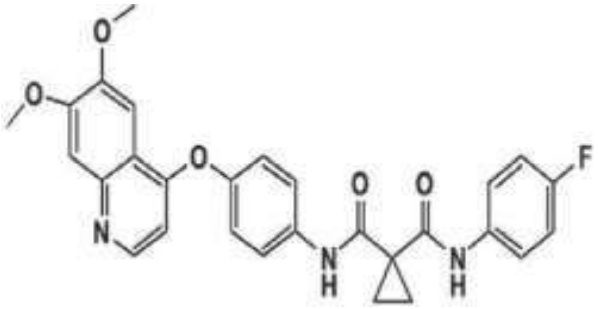

Fig. No. 1:- Structure of Cabozantinib

Table 2:- Drug profile of Nivolumab

Drug	Nivolumab
Synonym	Nivolumab
Category	Antineoplastic Agent
Molecular formula	$C_{6362}H_{9862}N_{1712}O_{1995}S_{42}$
Melting point	80-90°C

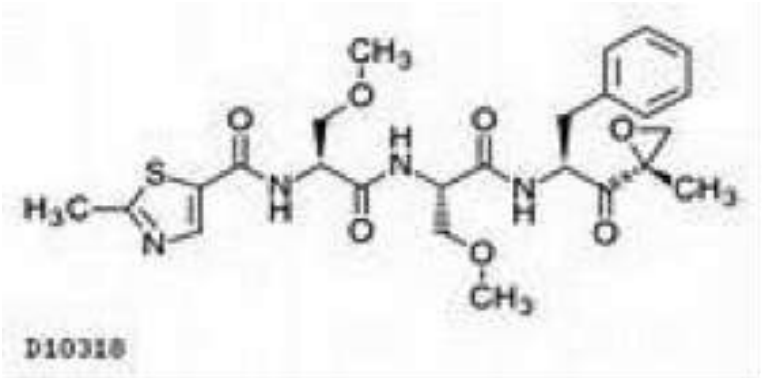

Fig. 2:- Structure of Nivolumab

Introduction to HPLC:

HPLC is also called high-pressure liquid chromatography since high pressure is used to increase the flow rate and efficient separation by forcing the mobile phase through at much higher rate. The pressure is applied using a pumping system. The development of HPLC from classical column chromatography can be attributed to the development of smaller particle sizes. Smaller particle size is important since they offer more surface area over the conventional large particle sizes. The HPLC is the method of choice in the field of analytical chemistry, since this method is specific, robust, linear, precise, accurate and the limit of detection is low and also it offers the following advantages.

Improved resolution of separated substances, Column packing with very small (3, 5 and 10 µm) particles, Faster separation times (minutes), Sensitivity Reproducibility, Continuous flow, detectors capable of handling small flow rates, Easy sample recovery, handling and maintenance.[29-31]

Materials and Methods:-

Table 3:- Instruments used

S. No.	Instruments and Glassware	Manufacturers
1	HPLC	WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA detector
2	pH meter	Lab India
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil
7	Digital ultra sonicator	Labman

Table 4:- Chemicals used

S. No.	Chemical	Supplier
1	Cabozantinib (Pure)	Sura labs
2	Nivolumab (Pure)	Sura labs
3	Water and Methanol for HPLC	LICHROSOLV (MERCK)
4	Acetonitrile for HPLC	Merck

HPLC Method Development:

Trails

Preparation of standard solution:

Accurately weigh and transfer 10 mg of Cabozantinib and Nivolumab working standard into a 10 ml of clean dry volumetric flasks add about 7 ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol. Further pipette 2.25 ml of the above Cabozantinib and 0.45 ml of the Nivolumab stock solutions into a 10 ml volumetric flask and dilute up to the mark with Methanol.

Procedure:

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH.

Mobile Phase Optimization:

Initially the mobile phase tried was Methanol: Water, Acetonitrile: Water with varying proportions. Finally, the mobile phase was optimized to Methanol and water in proportion 65:35 v/v respectively.

Optimization of Column:

The method was performed with various columns like C18 column, X- bridge column, Xterra. Phenomenex Luna C18 (4.6 x 150mm, 5 m) was found to be ideal as it gave good peak shape and resolution at 1 ml/min flow.

Validation Parameters

Specificity Study of Drug:

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Cabozantinib and 10 mg of Nivolumab working standard into a 10 ml of clean dry volumetric flasks, add about 7 mL of diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.03 ml of Cabozantinib and 3.0 ml of Nivolumab from the above stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluents.

Procedure:
The standard solution was injected five times and measured the area for all five injections in HPLC. The %RSD for thearea of five replicate injections was found to be within the specified limits.

Preparation of Sample Solution:
Take average weight of Tablet and crush in a mortar by using pestle and weigh 10 mg equivalent weight of Cabozantinib and Nivolumab sample into a 10 mL clean dry volumetric flask and add about 7 mL of diluent and sonicate to dissolve itcompletely and make volume up to the mark with the same solvent.

Further pipette 0.03 ml of Cabozantinib and 3.0 ml of Nivolumab from the above stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluents.

Preparation of Drug Solutions for Linearity:
Accurately weigh and transfer 10 mg of Cabozantinib and 10 mg of Nivolumab working standard into a 10 ml of clean dry volumetric flasks add about 7 mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (75 µg/ml of Cabozantinib & 15 µg/ml of Nivolumab):
Pipette out 0.01 ml of Cabozantinib and 1.0 ml of Nivolumab stock solutions was taken in a 10 ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – II (150 µg/ml of Cabozantinib & 30 µg/ml of Nivolumab):
Pipette out 0.02 ml of Cabozantinib and 2.0 ml of Nivolumab stock solutions was taken in a 10 ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – III (225 µg/ml of Cabozantinib & 45 µg/ml of Nivolumab):
Pipette out 0.03 ml of Cabozantinib and 3.0 ml of Nivolumab stock solutions was taken in a 10 ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – IV (300 µg/ml of Cabozantinib & 60 µg/ml of Nivolumab):
Pipette out 0.04 ml of Cabozantinib and 4.0 ml of Nivolumab stock solutions was taken in a 10 ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – V (375 µg/ml of Cabozantinib & 75 µg/ml of Nivolumab):
Pipette out 0.05 ml of Cabozantinib and 5.0 ml of Nivolumab stock solutions was taken in a 10 ml of volumetric flaskdilute up to the mark with diluent.

Procedure:
Inject each level into the chromatographic system and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate thecorrelation coefficient.

Precision Repeatability
Preparation of Cabozantinib and Nivolumab Product Solution for Precision:
Accurately weigh and transfer 10 mg of Cabozantinib and 10 mg of Nivolumab working standard into a 10 ml of clean dry volumetric flasks add about 7 mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.03 ml of Cabozantinib and 3.0 ml of Nivolumab from the above stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluents.

The standard solution was injected five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

Intermediate Precision:
To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.

Procedure:
DAY 1:
The standard solution was injected six times and measured the area for all six injections in HPLC. The %RSD for thearea of six replicate injections was found to be within the specified limits.

DAY 2:
The standard solution was injected six times and measured the area for all six injections in HPLC. The %RSD for thearea of six replicate injections was found to be within the specified limits.

Accuracy:
For preparation of 50% Standard stock solution:
Accurately weigh and transfer 10 mg of Cabozantinib and 10 mg of Nivolumab working standard into a 10 ml of clean dry volumetric flasks add about 7 ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.015 ml of Cabozantinib and 1.5 ml of Nivolumab from the above stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluents.

For preparation of 100% Standard stock solution:
Accurately weigh and transfer 10 mg of Cabozantinib and 10 mg of Nivolumab working standard into a 10 ml of clean dry volumetric flasks add about 7 ml of Diluents and sonicate to dissolve it completely and make volume up to the markwith the same solvent. (Stock solution)

Further pipette 0.03 ml of Cabozantinib and 3.0 ml of Nivolumab from the above stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluents.

For preparation of 150% Standard stock solution:
Accurately weigh and transfer 10 mg of Cabozantinib and 10 mg of Nivolumab working standard into a 10 ml of clean dry volumetric flasks add about 7 ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.045 ml of Cabozantinib and 4.5 ml of Nivolumab from the above stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluents.

Procedure:
Inject the three replicate injections of individual concentrations (50%, 100%, 150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found, and Amount added for Cabozantinib and Nivolumab and calculate the individual recovery and mean recovery values.

Robustness:
The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results.

For preparation of Standard solution:
Accurately weigh and transfer 10 mg of Cabozantinib and 10 mg of Nivolumab working standard into a 10 ml of clean dry volumetric flasks, add about 7 ml of diluents and sonicate to dissolve it completely and make volume up to the markwith the same solvent. (Stock solution)

Further pipette 0.03 ml of Cabozantinib and 3.0 ml of Nivolumab from the above stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluents.

Effect of Variation of flow conditions:
The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1 ml/min, remaining conditions are same. 10 µl of the above sample was injected and chromatograms were recorded.

Effect of Variation of mobile phase organic composition:
The sample was analyzed by variation of mobile phase i.e., Methanol: Water was taken in the ratioand 50:50, 60:40 instead (65:35), remaining conditions are same. 10 µl of the above sample was injected and chromatograms were recorded.

Results and Discussion of System Suitability:-

Chromatographic conditions:
The method was performed with various columns like C18 column, X- bridge column, Xterra. Phenomenex Luna C18 (4.6 x 250 mm, 5 mm) was found to be ideal as it gave good peak shape and resolution at 1 ml/min flow, equilibrated with Methanol and Water (65:35 v/v) as a mobile phase. The run time was 6 min and here the peaks were separated and showed better resolution. Conditions of optimized chromatography are shown in table no. 5:

Table No. 5:- Optimized Chromatographic Conditions

Mobile phase	Methanol: Water (65:35 v/v)
Wavelength	220 nm
Flow rate	1 ml/min
Run time	7 min
Temperature of the column	40ºC
Injection volume	10 µl
Column	Phenomenex Luna C18 (4.6×250mm) 5µ

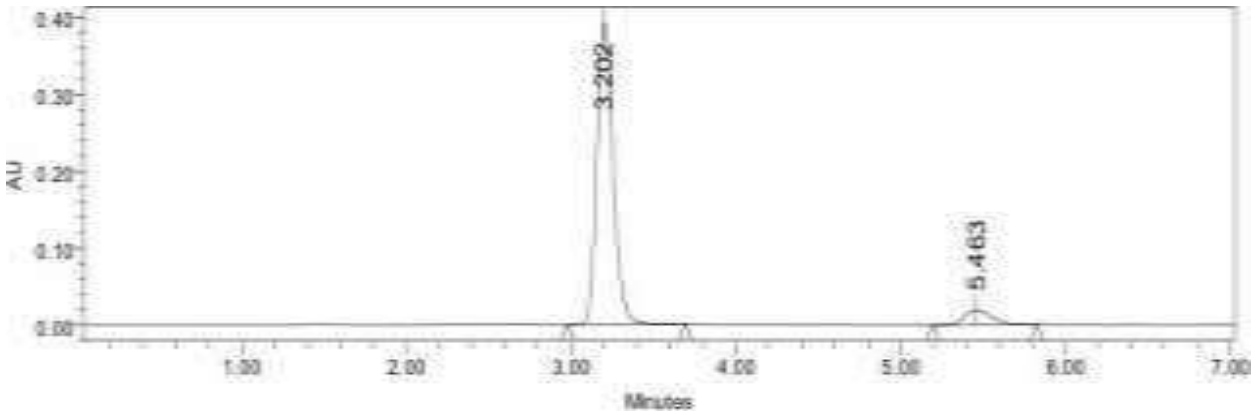

Fig. No. 3:- Optimized chromatogram of Cabozantinib (RT = 3.202 min) & Nivolumab (RT = 5.463 min)

Specificity:
There were no other components present at the elution time for Cabozantinib and Nivolumab. As seen in figure 3, the blank chromatogram is present.

Linearity:
The linearity range was found to be 75-375 µg/ml of Cabozantinib, 15-75 µg/ml of Nivolumab and chromatograms are shown in table no. 6.

Table No. 6:- Linearity Data of Cabozantinib and Nivolumab

S. No.	Cabozantinib		Nivolumab	
	Working conc. (µg/ml)	Peak Area	Working conc. (µg/ml)	Peak Area
1	75	909889	15	61953
2	150	1583641	30	130213
3	225	2395378	45	198697
4	300	3185089	60	267002

5	375	3943725	75	321658
Correlation coefficient (r)		0.9992	Correlation Coefficient (r)	0.999
Slope		10225	Slope	4365
Intercept (c)		102808	Intercept (c)	454

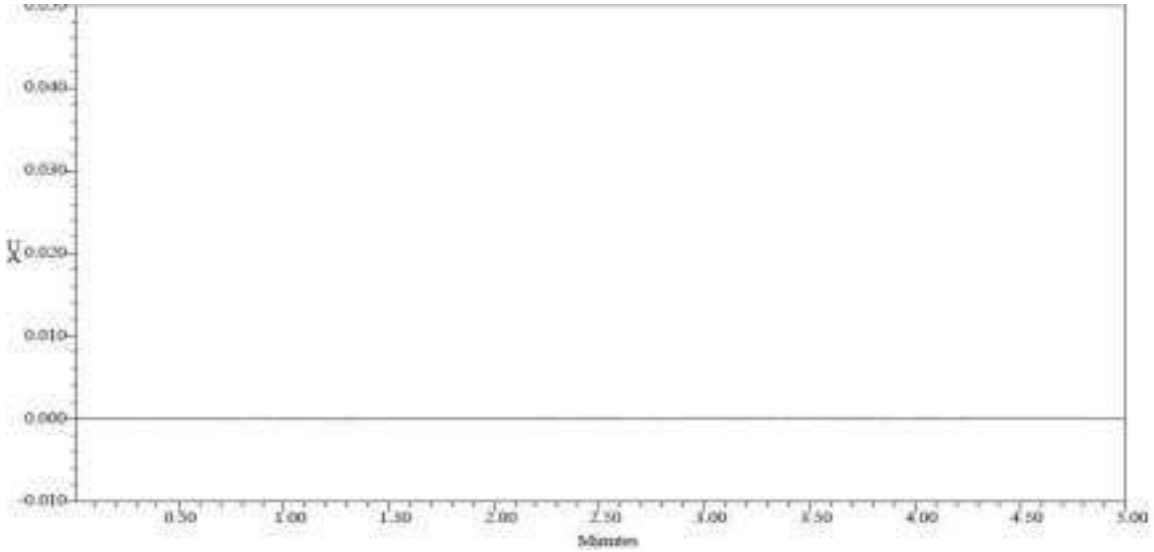

Fig. No. 4:- Chromatogram of blank

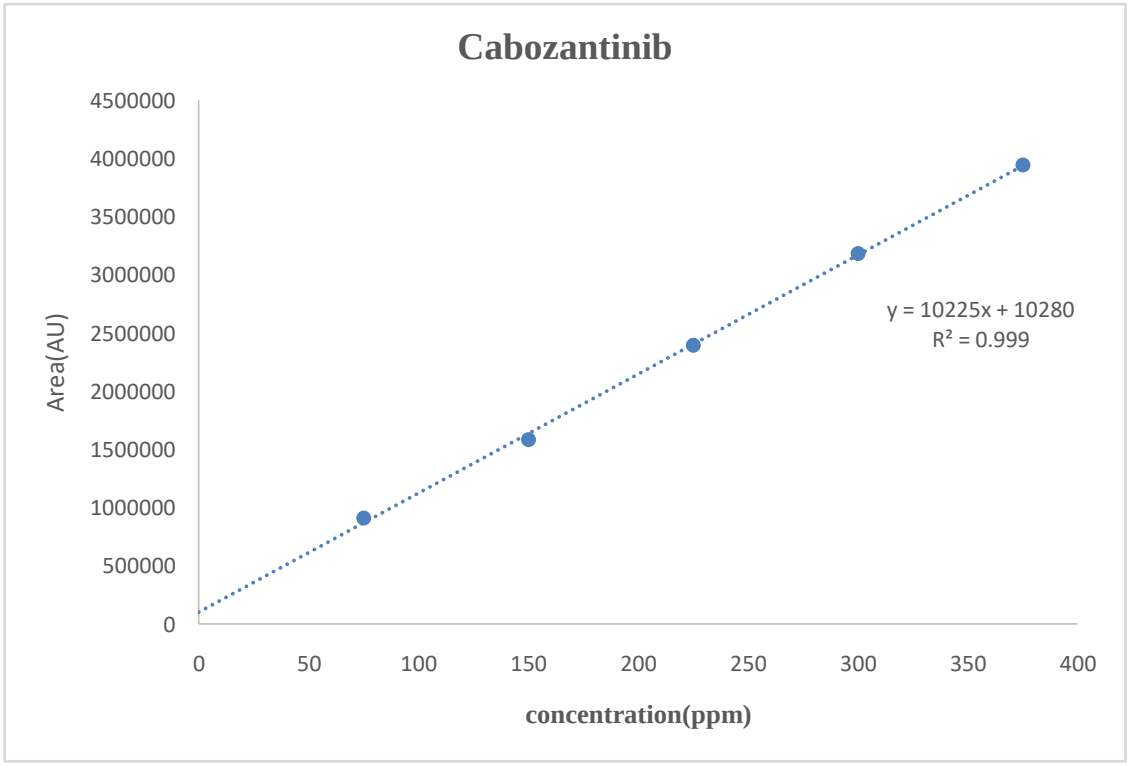

Fig. No. 5:- Calibration plot of Cabozantinib

Precision:

Precision of the method was carried out for both sample and standard solutions as described under experimental work. The corresponding chromatogram and results are shown in table no. 7.

Table No. 7:- Results of method precision

S. No.	Cabozantinib		Nivolumab	
	Retention time (min)	Peak Area	Retention time (min)	Peak Area
1	3.213	2397164	5.441	198464
2	3.253	2391741	5.442	193643
3	3.297	2371846	5.409	196462
4	3.215	2361748	5.520	194644
5	3.254	2371649	5.424	198464
Mean		2378830		196335.4
Std. Dev.		14958		2190.191
% RSD		0.628797		1.115536

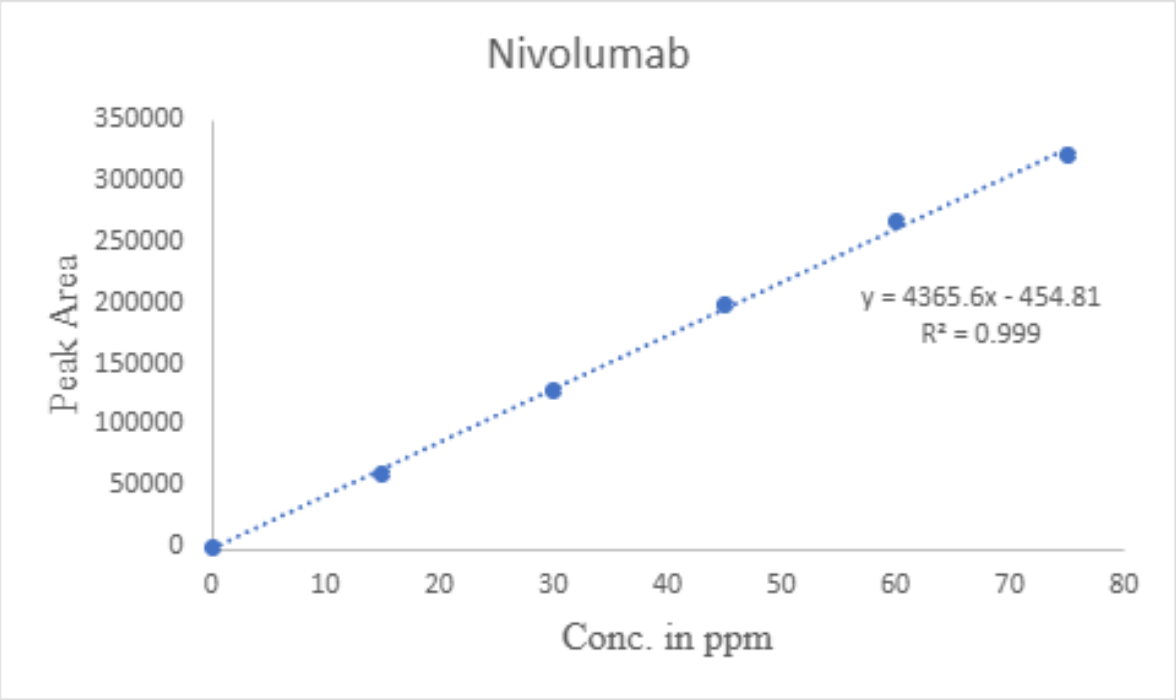

Fig. No. 6:- Calibration plot of Nivolumab

Table No. 8:- Results of intermediate precision

S. No.	Cabozantinib		Nivolumab	
	Retention time (min)	Peak Area	Retention time (min)	Peak Area
1	3.211	2389572	5.411	197284
2	3.211	2391847	5.410	197849
3	3.210	2319472	5.420	196572
4	3.212	2306842	5.423	195028
5	3.211	2375972	5.419	199474
6	3.297	2396746	5.409	197482
Mean		2363409		197281.5
Std. Dev.		39730.83		1466.354
%RSD		1.681082		0.74328

Accuracy:

Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the %recovery was calculated.

Table No. 9:- The Accuracy results for Cabozantinib and Nivolumab

Accuracy level	Cabozantinib			Nivolumab		
	Amount added (µg/ml)	Amount found (µg/ml)	% Recovery	Amount added (µg/ml)	Amount found (µg/ml)	%Recovery
50%	112.5	112.4	99.6	22.5	22.4	99.9
100%	225	225	100	45	45	100
150%	337.5	332.5	98.5	67.5	66.8	99
Mean% Recovery	99.3			99.6		

Limit of detection and Limit of quantification (LOD & LOQ):

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detectedbut not necessarily quantitated as an exact value. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

Table No. 10:- LOD & LOQ data for Cabozantinib and Nivolumab

Drug	LOD (µg/ml)	LOQ (µg/ml)
Cabozantinib	12.5	38.1
Nivolumab	3.7	11.4

Robustness

The robustness was performed for the flow rate variations from 0.9 ml/min to 1.1 ml/min and mobile phase ratio variationfrom more organic phase to less organic phase ratio for Cabozantinib and Nivolumab. The method is robust only in less flow condition and the method is robust even by change in the Mobile phase ± 5 %.

Table No. 11:- Robustness data for Cabozantinib and Nivolumab

Parameter used for sample analysis	Cabozantinib		Nivolumab	
	Retention Time	Tailing factor	Retention time	Tailing factor
Actual Flow rate of 1.0 ml/min	3.202	1.2	5.463	1.1
Less Flow rate of 0.9 ml/min	3.639	1.2	6.250	1.1
More Flow rate of 1.1 ml/min	2.859	1.1	4.863	1.2
Less organic phase	3.460	1.2	6.196	1.1
More Organic phase	3.022	1.1	5.010	1.2

Conclusion:-

The study is focused to develop and validate RP - HPLC method for estimation of Cabozantinib and Nivolumab in bulk and tablet dosage form. For routine analytical purpose it is desirable to establish methods capable of analysing huge number of samples in a short time period with good robustness, accuracy and precision without any prior separation steps. HPLC method generates large amount of quality data, which serve as highly powerful and convenient analytical tool. The method shows good reproducibility and good recovery. From the specificity studies, it was found that the developedmethods were specific for Cabozantinib and Nivolumab.

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