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A New Selective Separation method development and Validation of Cabozantinib and Nivolumab Using HPLC

Dr. T N V S S Satyadev
Dept of Chemistry, P B Siddhartha College of Arts and Science, Vijayawada, AP.

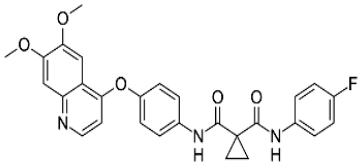
Abstract:

A reliable, accurate and simple RP-HPLC method was developed for the simultaneous estimation of Cabozantinib and Nivolumab, validated according to ICH guidelines. A column of X-bridge phenyl (150x4.6mm, 3.5µm) with a flow rate of 1ml/min was used. The combination of 0.1% Tri ethyl amine and Acetonitrile in 70:30 ratios was used as a mobile phase. Cabozantinib and Nivolumab peaks were eluted at a retention time of 4.358min, 7.744min respectively. The total run time was 10min. Standard solutions were prepared by dissolving in acetonitrile first and then make up to the mark with mobile phase. The method shows a good linearity in the concentration range of 6-90µg/ml of Cabozantinib and 4-60µg/ml of Nivolumab with correlation coefficient 0.999. This method was validated in terms of specificity, linearity, accuracy, LOD, LOQ, robustness and forced degradation.

Key words: RP-HPLC, Nivolumab, Cabozantinib, Validation.

1. INTRODUCTION

Cabozantinib, sold under the brand names Cometriq and Cabometyx among others, is a medication used to treat medullary thyroid cancer [1, 2], renal cell carcinoma [3], and hepatocellular carcinoma [4, 5]. It is a small molecule inhibitor of the tyrosine kinases c-Met and VEGFR2, and also inhibits AXL and RET. It was discovered and developed by Exelixis Inc. Cabozantinib is used in two forms. A capsule form is used since 2012, to treat medullary thyroid cancer and a tablet form is used since 2016, as a second line treatment for renal cell carcinoma. Cabozantinib has not been tested in pregnant women; it causes harm to fetuses in rodents [6]. Pregnant women should not take this drug, and women should not become pregnant while taking it. It is not known if cabozantinib is excreted in breast milk [7]. The drug should be used with caution in people with a history of heart rhythm problems [8], including long QT interval.



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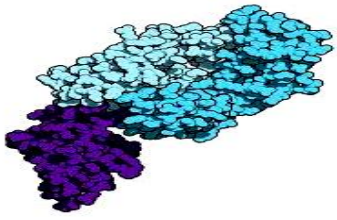

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Fig. No. 1: Structure of (A) Cabozantinib and (B) Nivolumab

Nivolumab, sold under the brand name Opdivo, is a medication used to treat a number of types of cancer. This includes melanoma, lung cancer [9, 10], renal cell carcinoma, Hodgkin lymphoma [11], head and neck cancer, colon cancer [12], and liver cancer [13]. It is used by slow injection into a vein. Common side effects include tiredness [14], rash, liver problems, muscles pains, and cough. Severe side effects may include immune-related lung, intestinal, liver, kidney, skin, or endocrine problems. Use during pregnancy may harm the baby and use when breastfeeding is not recommended. Nivolumab is a human IgG4 monoclonal antibody that blocks PD-1. It is a type of immunotherapy and works as a checkpoint inhibitor, blocking a signal that prevents activation of T cells from attacking the cancer.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Acetonitrile, tri ethyl amine (TEA), water and methanol were purchased from Merck (India) Ltd. Worli, Mumbai, India. All API's of Cabozantinib, Nivolumab as reference standards were procured from Dr Reddys Laboratories, Hyderabad.

2.2 Instrumentation

Waters alliance e-2695 chromatographic system consisting of quaternary pump, PDA detector-2996 and chromatographic software Empower-2.0 was used.

2.3 Preparation of stock and working standards

Preparation of standard solution: Accurately weighed 60mg of Cabozantinib and 40mg of Nivolumab working standards were transferred into 100ml volumetric flask. Add approximately 70ml of diluents and sonicated for 15min to dissolve the components, after 15min. makeup to the mark with diluents. Further diluted 5ml of the above solution to 50ml volumetric flask and diluted to volume with diluents.

Preparation of Sample solution: 2 Cabozantinib tablets were weighed and crush the 2 tablets into powder form, take the sample equivalent to 60mg of Cabozantinib and 40 mg of Nivolumab injection were transferred into a 100ml volumetric flask and add 70ml of diluents and sonicated for 50mins to dissolve the components and then diluted up to

the mark with diluents. Further dilute 5ml of the above solution to 50ml with diluents and it was filtered through 0.45µ nylon syringe filter.

2.4 Method Validation

The analytical method was validated as per ICH Q2 (R_1) guidelines for the parameters like system suitability, specificity, accuracy, precision, linearity, robustness, LOD, LOQ, forced degradation and stability.

2.4.1 System suitability

System suitability parameters like USP plate count, USP tailing and %RSD were measured and found to be within the limits.

2.4.2 Accuracy

Accuracy is the closeness of the test results obtained by the method to the true value. It was studied at three different concentration levels (50%, 100% and 150% levels). Minimum three injections were given in each level and percentage of recovery, % RSD was calculated.

2.4.3 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of other components (impurities, degradates or excipients), which may be expected to be present in the sample and standard solution. It was checked by examining the chromatograms of blank samples and samples spiked with Cabozantinib and Nivolumab.

2.4.4 Precision

In this method precision was evaluated as system precision, method precision and intermediate precision.

In system precision six replicate standard solutions of Cabozantinib and Nivolumab were analyzed and %RSD was calculated.

In method precision six preparations with sample were injected and %RSD, % recovery were calculated. The intraday and inter-day precision study was conducted for both Cabozantinib and Nivolumab.

2.4.5 Linearity and range

Linearity was conducted by preparing different standard solutions of Cabozantinib and Nivolumab at different concentration levels. The standard solutions were prepared in the concentration range of 6-90µg/ml of Cabozantinib and 4-60µg/ml of Nivolumab. Each concentration was injected into the HPLC system and record the areas obtained. Plot a graph between area taken on Y-axis and concentration on X-axis.

2.4.6 LOD and LOQ

LOD was measured by diluting the standard solution of Cabozantinib and Nivolumab and determining the concentration was response of sample peaks are three times the noise peak. LOQ was measured by diluting the standard solution of Cabozantinib and Nivolumab and determining the concentration was response of sample peaks are ten times the noise peak.

2.4.7 Stress degradation studies

Forced degradation studies were used to evaluate the specificity of the method. The degradation peaks should be well separated from each other and the resolution between the peaks should be at least 1.0 and peak purity of the principle peaks shall pass. Forced degradation studies were performed by different types of stress conditions to obtain the degradation of about 20%.

2.4.8 Robustness

In robustness the method was determined by making slight changes in the flow rate ±20%, organic phase ±10%, wave length by ±5nm.

3. RESULTS

3.1 Method development and optimization

The most suitable isocratic condition to resolve Cabozantinib and Nivolumab with X-bridge phenyl column, after the chromatographic conditions were optimized for specificity, resolution and retention time was a mobile phase consisting of 0.1% Tri ethyl amine and Acetonitrile in the ratio of 70:30. When a higher percentage of mobile phase was used, the resultant chromatogram had an increase either in back ground noise or peaks indicating the tailing effect. Thus based on the above mentioned parameters, Cabozantinib and Nivolumab were eluted at a retention time of 4.358 min and 7.744 min respectively. Table 1 depicts the chromatographic parameters applied for the method.

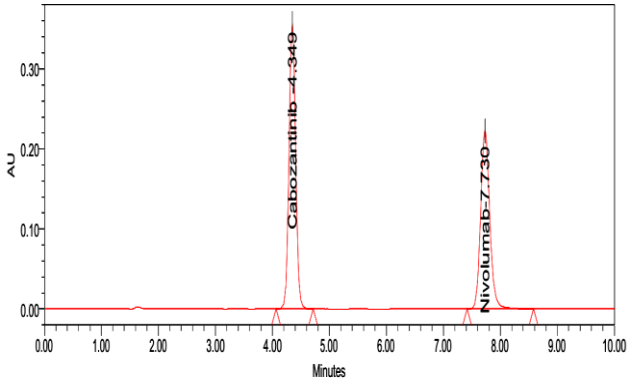


Fig. No. 2: Representative chromatogram of Cabozantinib and Nivolumab

Table 1: HPLC isocratic method for Cabozantinib and Nivolumab

S. No.	Parameter	Method Conditions
1	Column	X-bridge phenyl 150x4.6mm, 3.5µ
2	Flow rate	1 ml/min
3	Wave length	222nm
4	Injection Volume	10µl
5	Run time	10 min
6	Mobile phase	0.1% TEA: ACN 70:30

3.2 Method Validation

The method was validated according to the validation of analytical procedures provided in the ICH guidelines and draft guidance for the industry, analytical procedures and method validation.

System suitability

The standard solution was introduced into the HPLC system and found that system suitability parameters are within the limits. The %RSD was calculated to standard peak areas. The system precision results were tabulated in table 2 and the chromatogram of standard was exhibited in the figure 3.

Table 2: Results of system precision

S. No	System suitability parameter	Acceptance criteria	Drug Name	
			Cabozantinib	Nivolumab
1	% RSD	NMT 2.0	0.71	0.63
2	USP Tailing	NMT 2.0	1.11	1.24
3	USP Plate count	NLT 3000	4236	7518

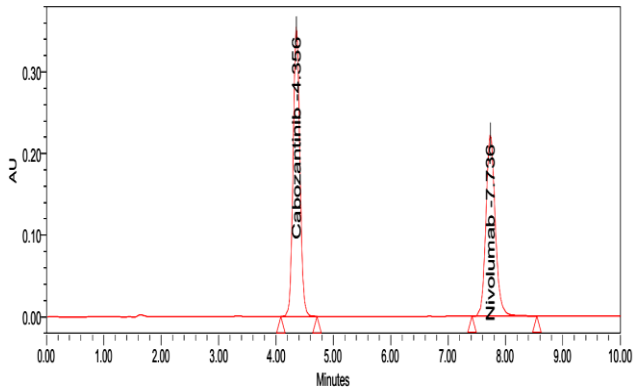


Fig. No. 3: Chromatogram of standard

Specificity

In specificity samples were prepared by adding equivalent weight of API and placebo with test concentration and then injected into HPLC system. Interference was not found for the chromatograms of placebo solution, empty cell solution and impurities at the retention time of Cabozantinib and Nivolumab.

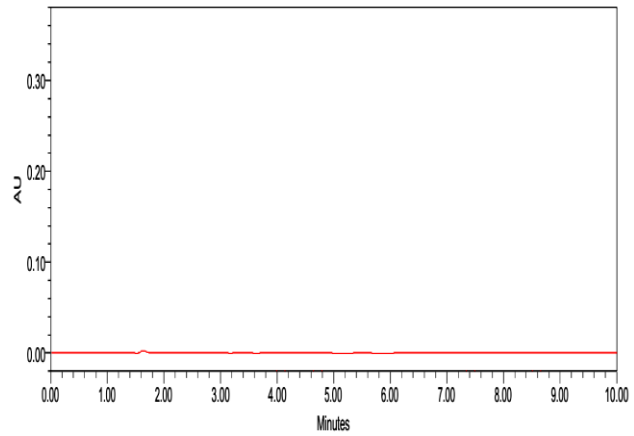


Fig. No. 4: Chromatogram of blank

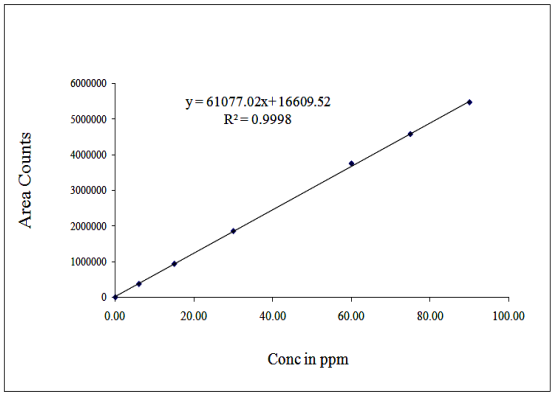
Linearity

Cabozantinib linearity concentration was prepared in the range of 6-90 µg/ml. The regression equation was found to be Y= 61077.02x+16609.52 and correlation coefficient was 0.9998.

Nivolumab linearity concentration was prepared in the range of 4-60µg/ml. The regression equation was found to be Y=60932.39x+6911.06 and correlation coefficient was 0.9999.

Table 3: Results of linearity

S. No.	Cabozantinib		Nivolumab	
	Conc. (µg/ml)	Area	Conc. (µg/ml)	Area
Linearity-1	6.00	369521	4.00	239587
Linearity-2	15.00	935634	10.00	627481
Linearity-3	30.00	1856487	20.00	1230625
Linearity-4	60.00	3754128	40.00	2451785
Linearity-5	75.00	4582517	50.00	3065247
Linearity-6	90.00	5475236	60.00	3645213
Slope	61077.02		60932.39	
Intercept	16609.52		6911.06	
CC	0.9998		0.9999	



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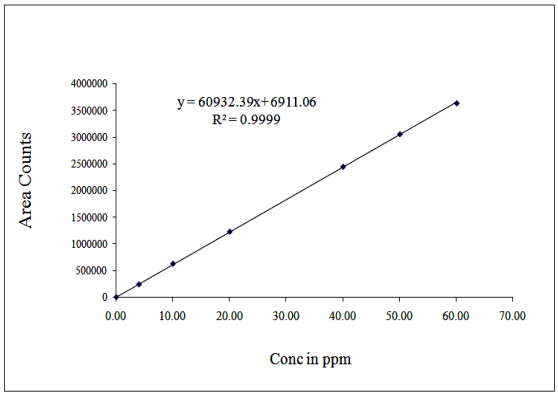

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Fig. No. 5: Linearity plots of (A) Cabozantinib and (B) Nivolumab

Robustness

In robustness there is a small deviation in flow rate (±0.2ml) and organic solvent (±10%) in their chromatic condition and observed that there is no significant change in %RSD.

Table 4: Results of robustness

S.No	Parameter name	% RSD for purity	
		Cabozantinib	Nivolumab
1	Flow (0.8ml/min)	0.36	0.21
2	Flow (1.2ml/min)	0.27	0.39
3	Organic solvent (+10%) (33:67)	0.44	0.42
4	Organic solvent (-10%) (27:73)	0.96	0.76

Precision

Precision of the method was established by injecting test preparation and tested through the complete analytical procedure from sample preparation to the final result.

Table 5: Results of method precision

Analyte	Std Conc.	%RSD
Cabozantinib	60	0.33
Nivolumab	40	0.75

Intermediate Precision

Six replicates of a sample solution was analyzed on a different day, different analyst and different RSD values.

Table 6: Results of Intermediate precision

Analyte	Std. Conc.	%RSD
Cabozantinib	60	0.48
Nivolumab	40	0.91

Limit of Detection and Limit of Quantification (LOD & LOQ)

The LOD concentrations of Cabozantinib and Nivolumab were 0.075µg/ml and 0.05µg/ml and LOQ concentrations of Cabozantinib and Nivolumab was 0.248µg/ml and 0.165µg/ml respectively.

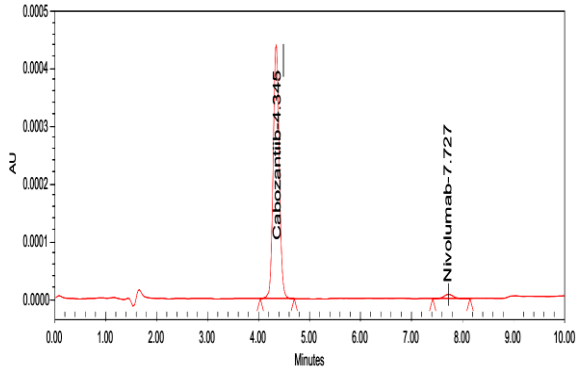

Fig. No. 6: Chromatogram of LOD

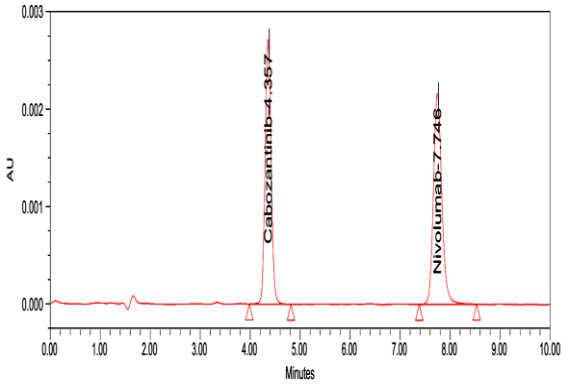

Fig. No. 7: Chromatogram of LOQ

Accuracy

Accuracy was determined by recovery studies which were carried out in three different concentration levels (50%, 100% and 150%). APIs with concentration 30, 60 and 90µg/ml of Cabozantinib and 20, 40 and 60g/ml were prepared. The percentage recovery values were found to be in the range of 98-102%.

Table 7: Results of Accuracy of Cabozantinib

S. No.	% Level	% Recovery	Ave %Recovery
1	50	99.4	98.9
2		98.5	
3		98.9	
4	100	99.2	99.6
5		99.4	
6		100.3	
7	150	99.5	99.5
8		99.3	
9		99.7	

Table 8: Results of Accuracy of Nivolumab

S. No.	% Level	% Recovery	Ave % Recovery
1	50	98.3	98.5
2		98.3	
3		98.8	
4	100	100.9	100.6
5		100.8	
6		100.0	
7	150	99.1	99.2
8		98.7	
9		99.8	

Degradation effects and its characterization

Cabozantinib and Nivolumab sample was subjected into various forced degradation conditions to effect partial degradation of the drug. Forced degradation studies were performed to show the method is suitable for degraded products. Moreover, the studies provide information about the conditions in which the drug is unstable so that measures can be taken during formulation to avoid potential instabilities.

Acid Degradation

Initially Cabozantinib and Nivolumab were studied in 0.1N HCl there is no degradent peaks are formed. When the strength of acid was increased to 1N HCl and heated at 60°C for 30mins, 15% of Cabozantinib and 15% of Nivolumab.

Alkali Degradation

Initially Cabozantinib and Nivolumab was studied in 0.1N NaOH there is no degradent peaks are formed. When the strength of alkali was increase to 1N NaOH and heated at 60°C for 30mins, 12% of Cabozantinib and 12% of Nivolumab degradation was observed.

Oxidation

Initially Cabozantinib and Nivolumab were studied in 10% peroxide there is no degradent peaks are formed. Then the strength of peroxide was increased to 30% and reflux for 3hrs 17% of Cabozantinib and 20% of Nivolumab degradation was observed.

Reduction

First trial of Cabozantinib and Nivolumab were studied in 10% sodium bi sulphate solution was used to study the reduction degradation there was no degradent was formed. After that the above solution refluxed for 3hrs 16% of Cabozantinib and 15% of Nivolumab was observed.

Thermal Degradation

For the first trial of thermal degradation sample was exposed at 105°C for 3hrs and the exposed sample was analyzed there was no degradent peaks are formed. After that the above solution was refluxed for 3hrs 15% of Cabozantinib and 12% of Nivolumab degradation was observed.

Photolytic Degradation

For the first trial of photolytic degradation sample was exposed in UV light for 6Hrs and the exposed sample was analyzed there was no degradent peaks are formed. After that the exposed sample was refluxed for 3hrs 11% of Cabozantinib and 15% of Nivolumab degradation was observed.

Table 9: Forced degradation results of Cabozantinib and Nivolumab

Degradation Condition	% Degradation	
	Cabozantinib	Nivolumab
Unstressed Degradation	99.9	99.9
Acid Degradation	13.4	12.7
Alkali Degradation	12.9	12.1
Peroxide Degradation	12.2	13.5
Reduction Degradation	11.5	11.6
Thermal Degradation	10.9	11.0
Photolytic Degradation	10.1	10.4

4. DISCUSSION

In the present study, to separate Cabozantinib and Nivolumab we use reverse phase HPLC, X-bridge phenyl column, 0.1% Tri ethyl amine and acetonitrile (70:30) as mobile phase. The reliability, accuracy and precision within the ICH and FDA limits for the method validation of analytical samples. In addition, analysis of the marketed preparation of Cabozantinib and Nivolumab with the validated assay methods showed that the drug contents eluted with no interfering peaks generated by the excipients in the marketed products. Results for robustness and the method were found to remain unaffected by changing the method parameters.

5. CONCLUSION

A validated HPLC method for the assay of Cabozantinib and Nivolumab was developed. The degradation behaviour of the drug was investigated under hydrolysis (acid, base and neutral), oxidation, photolysis and thermal stress conditions.

An isocratic RP-HPLC method for the determination of Cabozantinib and Nivolumab was developed and is precise and reliable. The regression line equation is capable of reliably predicting the drug concentration in the range of 6-90 µg/ml of Cabozantinib and 4-60 µg/ml of Nivolumab respectively, from the peak area obtained. The method was successfully validated and allowed the reliable, sensitive, robust and specific detection of Cabozantinib and Nivolumab in a marketed preparation.

Conflicts Of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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