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Simultaneous Method Development and Validation For Estimation of Nivolumab and Cabozantinib In Bulk and Pharmaceutical Dosage Form by RP-HPLC Method

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ABSTRACT

A simple, precise, and accurate method was developed for the simultaneous estimation of Nivolumab (NVM) and Cabozantinib (CBZ) in tablet dosage form. The chromatogram was analyzed through a Phenomenex C18 150 mm (4.6 x 150 mm, 5 m) for chromatogram processing. A mobile phase containing formic acid: methanol (50:50) was pumped through the column at a spurge flow of 1.0 mL/min. The column temperature was upheld at 30°C. The quantification was done at 260.0 nm. The elution time of NVM and CBZ was found to be 2.243 min and 2.953 min, respectively. The validation for the developed method was performed and all the parameters were found within the specified limits. The standard curve results represent a correlation coefficient of more than 0.999. The %RSD of NVM and CBZ were found to be 0.9 and 0.7, respectively. % Recovery was achieved as 99.88% and 99.66% for NVB and CBZ, respectively. The NVM and CBZ regression equations yielded LOD and LOQ values of 0.63, 1.91, and 0.08, 0.24 respectively. The equation of Nivolumab y is 39306x + 12173 while the Cabozantinib y is 34894x + 1139.7. With all the parameters under the criteria, the developed method for the simultaneous estimation of Nivolumab and Cabozantinib can be successfully applied for regular quality control approaches.

Keywords: Nivolumab, Cabozantinib, Method Development, RP-HPLC, Validation

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INTRODUCTION

Nivolumab (Opdivo) helps shrivel growths, assists patients with cutting-edge melanoma to live longer, and diminishes the gamble of melanoma returning after a medical procedure. It is additionally endorsed for adjuvant treatment. It is utilized to treat progressed melanoma that is unresectable or has spread to organs and different pieces of the body [1]. Moreover, NVB is likewise utilized as adjuvant treatment, that is to say, treatment after complete careful resection of melanoma to lessen the gamble of the melanoma returning. NVB blocks the movement of a particle called PD-1[2], a protein that keeps Lymphocytes from perceiving and going after kindled tissues and malignant growth cells. PD-1 can fool your invulnerable framework into disregarding melanoma cells as typical cells.

Cabozantinib is an antineoplastic (cancer) drug that inhibits the growth of cancer cells until they are eventually destroyed by the body [3]. It is used to treat advanced kidney cancer, hepatocellular carcinoma, differentiated thyroid cancer[4] that has spread to other parts of the body, and hepatocellular carcinoma in patients who have previously received treatment with other drugs (such as sorafenib)[5]. For advanced kidney carcinoma, it is additionally used in conjunction with nivolumab as a first-line therapy.

From the literature survey, it was found that determination of NVM and CBZ were estimated by analytical methods such as Spectrophotometry methods by simultaneous determination of Nivolumab in combination with Cabozantinib [6], by RP-HPLC technique, A simple, sensitive and rapid chromatographic method was developed and validated for simultaneous quantification of Cabozantinib and Nivolumab in rat plasma using Alectinib as internal standard [7]. A simple, specific, accurate, and stability-indicating reversed-phase high-performance liquid chromatographic (RP-HPLC) method was developed for the estimation of Cabozantinib.[8] A reliable, accurate, and simple RP-HPLC method was developed for the simultaneous estimation of Cabozantinib and Nivolumab, validated according to ICH guidelines [9].

MATERIALS AND METHOD

Materials:

Nivolumab and Cabozantinib pure drugs (API), Combination NVB and CBZ Synthetic Formulation (Rhodes Pharmaceuticals), Distilled water, Acetonitrile, Phosphate buffer, Methanol, Potassium dihydrogen orthophosphate buffer, Ortho-phosphoric acid. All the above chemicals and solvents are from Rankem Pharmaceuticals.

Instruments:

Electronics Balance-Denver, pH meter -BVK enterprises, India, Ultrasonicator - BVK enterprises, WATERS HPLC 2695 SYSTEM equipped with quaternary pumps, Photo Diode Array detector and Autosampler integrated with Empower 2 Software, UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2 mm and 10 mm and matched quartz cells integrated with UV win 6 Software was used for measuring absorbances of Nivolumab and Cabozantinib solutions.

METHODS:

Chromatographic conditions:

Chromatographic analysis was done using isocratic elution, the column used was STD Phenomenex C18 150 mm (4.6 x 150 mm, 5 m) Mobile phase containing Buffer (formic acid): 50% Methanol: 50% elutant was pumped through the column at a spurge flow of 1.0 mL/min. The temperature was upheld at 30°C. The optimized wavelength selected was 260.0 nm.

Selection of Wavelength:

By Utilizing a PDA locator, the retention spectra of the arrangement of two drugs were examined in the UV light region 200-400nm spectra, the overlay of the two spectra consolidated at 260nm was chosen as the detection wavelength for the HPLC chromatographic technique.

Preparation of standard solution:

Accurately weighed and transferred 24 mg of NVB and 1 mg of CBZ working Standards into a 25 mL clean dry volumetric flask, added 3/4th volume of diluent, sonicated for 5 minutes, and made up to the final volume with diluents. (480 ppm of Nivolumab and 80 ppm of Cabozantinib). 1mL from the above two stock solutions was taken into a 10mL volumetric flask and made up to 10mL

Preparation of sample solution:

5 tablets of synthetic formulation were weighed and the average weight of each tablet was calculated, then the weight equivalent to 1 tablet (240mg) was transferred into a 100 volumetric flask, 5 mL of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters. 1 mL of filtered sample stock solution was transferred to a 10 mL volumetric flask and made up with a diluent.

Method validation:

For the chosen CZT and NVM drugs, this approach validates system suitability, linearity, precision, accuracy, robustness, LOD, LOQ, forced degradation, and stability.

System suitability parameters:

The system suitability parameters were determined by preparing standard solutions of NVM and CBZ, the solutions were injected six times and the parameters like peak tailing, resolution, and

USP plate count were determined. The % RSD for the area of six standard injections results should be no more than 2%.

Specificity:

The capacity to determine the analyte's specificity in the presence of other elements (impurities, degradation products, or excipients) that are likely present in the test and standard solution is known as specificity. The chromatograms of blank and spiked samples tampered with NVM and CBZ were examined to verify it.

Precision:

System, method, and intermediate precision were the metrics used to evaluate precision in this approach. Six replicate standard solutions of Nivolumab and Cabozantinib were examined in system precision, and the % RSD was computed.

Six preparations containing the test were injected in the method precision, and the percentage recovery and % RSD were computed. The study on intraday and inter-day precision was carried out for both NVM and CBZ.

Linearity:

Accurately Weighed and transferred 24 mg of Nivolumab and 4 mg of Cabozantinib working Standards into a 50 mL clean dry volumetric flask, add 3/4th volume of diluent, sonicated for 5 minutes, and makeup to the final volume with diluents. (480 ppm of Nivolumab and 80 ppm of Cabozantinib).

To achieve Linearity, various concentration levels of NVM and CBZ standard solutions were prepared, ranging from Nivolumab (12-72µg/mL) and Cabozantinib (2-12µg/mL). Each concentration was then injected into the HPLC system, and the areas obtained were recorded for each concentration.

Accuracy:

Three distinct concentration levels 50%, 100%, and 150% were investigated. Every level received a minimum of three injections, and the percentage of recovery and RSD were computed. Five tablets of synthetic formulation were weighed and the average weight of each tablet was calculated, then the weight equivalent to 1 tablet (240mg) was transferred into a 500 mL volumetric flask, and 50 mL of diluents were added, and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters (480µg/mL of Nivolumab and 80 µg/mL of Cabozantinib). 1mL from the above two stock solutions was taken into a 10mL volumetric flask and made up to 10mL. (48 ppm of Nivolumab and 8 ppm of Cabozantinib). The % Recovery for each level should be between 98.0 to 102 %.

Robustness:

To assess the robustness of the method, minor changes were made to the Temperature ($\pm 5^0$c), flow rate (±1.0%), and organic phase (±10%). Robustness conditions like Flow minus (0.9mL/min), Flow plus (1.1mL/min), mobile phase minus (55B:45A), mobile phase plus (65B:35A), temperature minus (25°C) and temperature plus(35°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. % RSD was within the limit.

LOD Sample Preparation:

0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10mL volumetric flasks and made up with diluents. From the above solutions 0.3mL each of Nivolumab and Cabozantinib, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluents

LOQ Sample Preparation:

0.25mL each from two standard stock solutions was pipetted out and transferred to two separate 10mL volumetric flasks and made up with diluent. From the above solutions 0.9mL each of Nivolumab and Cabozantinib, solutions respectively were transferred to 10mL volumetric flasks and made up with the same diluent.

Stability studies:

Various stress conditions were used to conduct forced degradation studies like Oxidation, acid, alkali, dry heat, and photo stability studies to evaluate the method specificity. Forced degradation was attempted as per the International Conference of Harmonization (ICH) guidelines Q1B.

Oxidation:

To 1 mL of stock solution of Nivolumab and Cabozantinib, 1 mL of 20% hydrogen peroxide (H_2O_2) was added separately. The solutions were kept for 30 min at 60^0c. For the HPLC study, the resultant solution was diluted to obtain 48µg/mL & 8µg/mL solution, and 10 µL was injected into the system and the chromatograms were recorded to assess the stability of the sample.

Acid degradation studies:

To 1mL of stock solution of Nivolumab and Cabozantinib, 1mL of 2N Hydrochloric acid was added and refluxed for 30 mins at 600c. The resultant solution was diluted to obtain 48µg/mL & 8µg/mL solution and 10 µL solutions were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Alkali degradation studies:

To 1 mL of stock solution of Nivolumab and Cabozantinib, 1 mL of 2N sodium hydroxide was added and refluxed for 30mins at 60^0c. The resultant solution was diluted to obtain 48µg/mL & 8µg/mL solution and 10 µL was injected into the system and the chromatograms were recorded to assess the stability of the sample.

Dry heat degradation studies:

The standard drug solution was placed in an oven at 105°C for 1 hr to study dry heat degradation. For the HPLC study, the resultant solution was diluted to 48µg/mL & 8µg/mL solution and 10µL were injected into the system and the chromatograms were recorded to assess the stability of the sample**.**

Photo-stability studies:

The photochemical stability of the drug was also studied by exposing the 480µg/mL Nivolumab & 80µg/mL Cabozantinib solution to UV Light by keeping the beaker in a UV Chamber for 1 day or 200Watt hours/m^2 in a photostability chamber. For the HPLC study, the resultant solution was diluted to obtain 48µg/mL & 8µg/mL solutions, and 10 µL was injected into the system and the chromatograms were recorded to assess the stability of the sample.

Neutral degradation studies:

Stress testing under neutral conditions was studied by refluxing the drug in water for 1hrs at a temperature of 60ºC. For the HPLC study, the resultant solution was diluted to 48µg/mL, & 8µg/mL, and 10 µL were injected into the system and the chromatograms were recorded to assess the stability of the sample**.**

RESULTS AND DISCUSSION:

System Suitability:

All the system suitability parameters were within the range and satisfactory as per ICH guidelines. CBZ and NVM were eluted at 2.243 min and 2.953 min respectively with good resolution. The plate count and tailing factor were very satisfactory, so this method was optimized and validated. All the system-suitable parameters were passed and were within the limits.

Table 1: System suitability parameters for Nivolumab and Cabozantinib

S.NO	Nivolumab			Cabozantinib			
Injection	RT (min)	USP Plate count	Tailing	RT (min)	USP Plate count	Tailing	Resolution
1	2.21	5852	1.25	2.79	8120	1.19	4.7
2	2.21	5572	1.27	2.79	8166	1.19	4.7
3	2.22	5818	1.24	2.80	8287	1.20	4.7
4	2.22	5504	1.29	2.80	8667	1.20	4.7
5	2.22	5478	1.25	2.80	8211	1.19	4.6
6	2.22	5515	1.24	2.80	8243	1.18	4.8

Specificity:

In this test method placebo, sample, and standard solution were analysed individually to identify the unwanted interferences. The active ingredients were separate from blank and their excipients and there was no placebo interference with the standard peak. Hence the method is specified as per the specificity study observation.

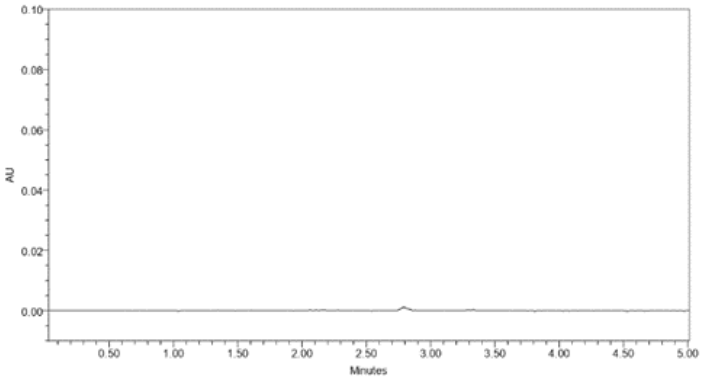


Figure 1: Chromatogram of Blank

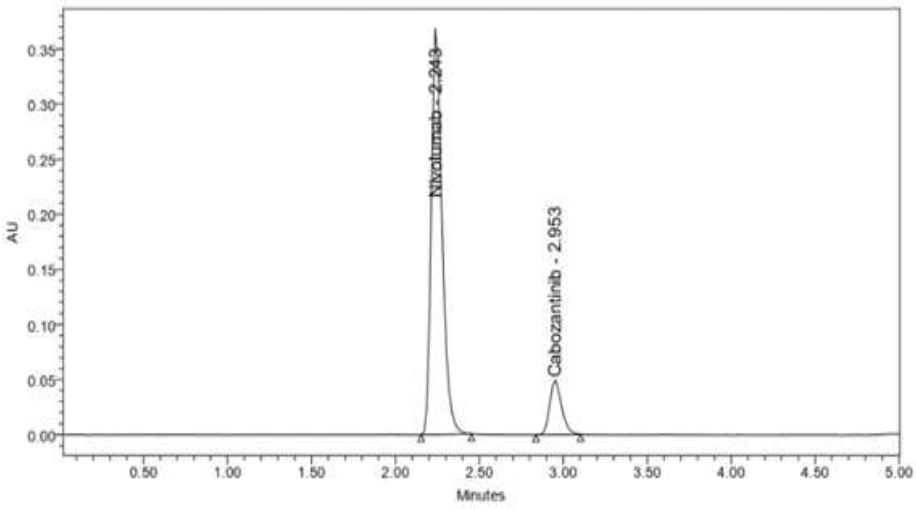


Figure 2: Standard Chromatogram of NVM and CBZ

Linearity:

Six linear concentrations of Nivolumab (12-72µg/mL) and Cabozantinib (2-12µg/mL) were injected in a duplicate manner. Average areas were mentioned above and linearity equations obtained for Nivolumab was y = 39306x + 12173 and for Cabozantinib was y = 34894x + 1139.7. The correlation coefficient obtained was 0.999 for both drugs.

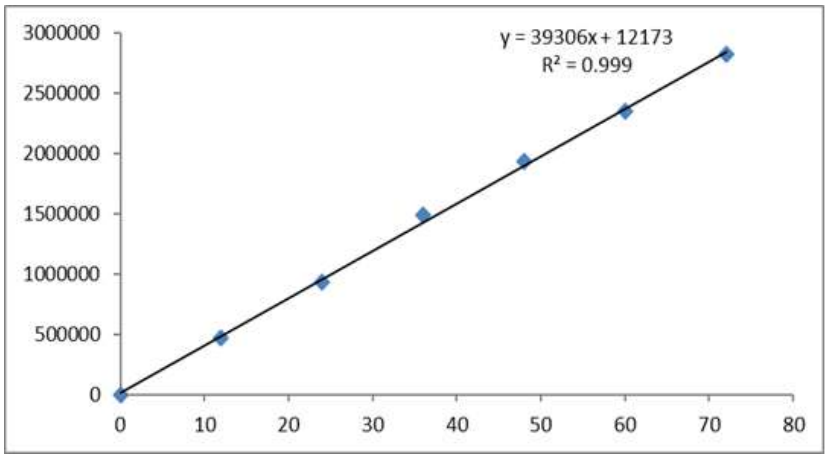


Figure 3: Calibration curve of NVM

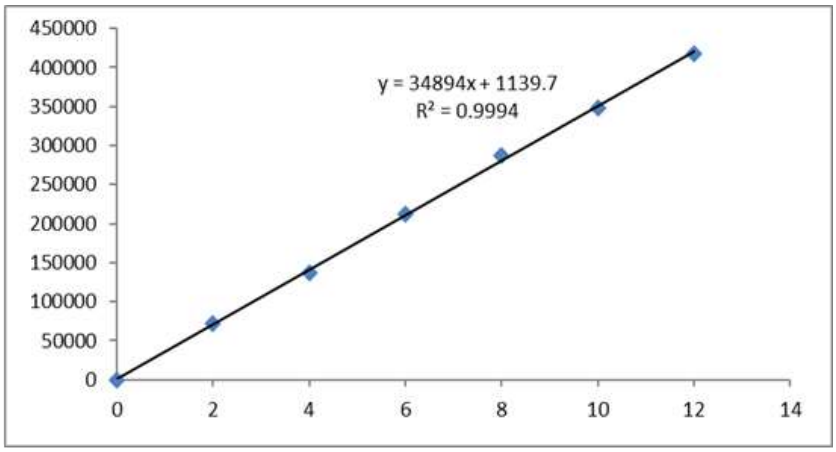


Figure 4: Calibration curve of CBZ 3.4 Precision:

From a single volumetric flask of working standard solution, six injections were given and the obtained areas were mentioned below. Average area, standard deviation, and % RSD were calculated for two drugs. % RSD obtained as 0.7% and 0.9% respectively for Nivolumab and Cabozantinib. As the limit of Precision was less than two the system precision was passed in this method. Repeatability and Inter-day Precision were also performed and passed within the limits.

Table 2: System Precision, Repeatability, and Inter-day Precision Table of NVM and CBZ

S.NO	System Precision (Area)		Repeatability (Area)		Intermediate Precision (Area)	
(N=6)	NVM	CBZ	NVM	CBZ	NVM	CBZ
I	1521624	234438	1529639	234138	1621975	268953
II	1529889	238304	1528862	237068	1616587	264222
III	1500407	233523	1519284	232840	1611224	267837
IV	1522792	232493	1520503	237179	1643416	269189
V	1527442	233354	1518919	233952	1615687	265004
VI	1517329	232549	1507599	233497	1625316	266746
Mean	1519914	234110	1520801	234779	1622368	266992
S.D	10531.7	2175.5	8041.3	1870.7	11437.7	2052.9
% RSD	0.7	0.9	0.5	0.8	0.7	0.8

Accuracy:

Three levels of Accuracy samples were prepared by the standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 99.57%, 100.90%and 100.00% for Nivolumab and 99.50%, 99.03%, and 100.35% for Cabozantinib respectively.

Table 3: Accuracy table of NVM and CBZ

	Level (%)	Spiked spl	Recovered	% Found	Mean %
NVM (N=6)	50	12	74.92	99.89	99.57
			74.47	99.29	
			74.66	99.54	
	100	24	151.45	100.97	100.90
			151.33	100.89	
			151.29	100.86	
	150	36	224.25	99.67	99.99
			225.30	100.13	
			225.44	100.19	
CBZ (N=6)	50	2	12.51	100.09	99.50
			12.38	99.07	
			12.42	99.36	
	100	4	24.73	98.92	99.04
			24.77	99.06	
			24.78	99.14	
	150	6	37.54	100.12	100.35
			37.65	100.41	
			37.70	100.54	

Sensitivity:

The method was evaluated according to the US FDA guidelines, and the LOD and LOQ concentrations for Nivolumab are 0.63 and 1.91 and the LOD and LOQ concentrations for Cabozantinib are 0.08 and 0.24.

Table 4: LOD and LOQ of NVM and CBZ

Molecule	LOD	LOQ
Nivolumab	0.63	1.91
Cabozantinib	0.08	0.24

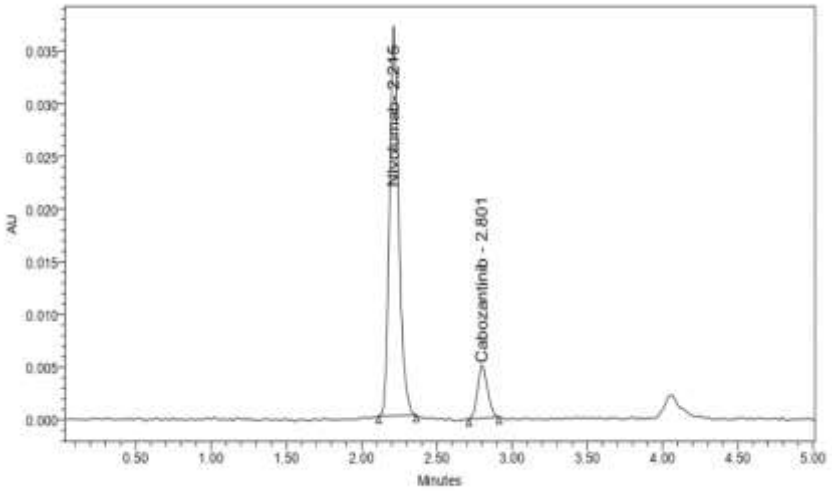


Figure 5: LOD of NVM and CBZ

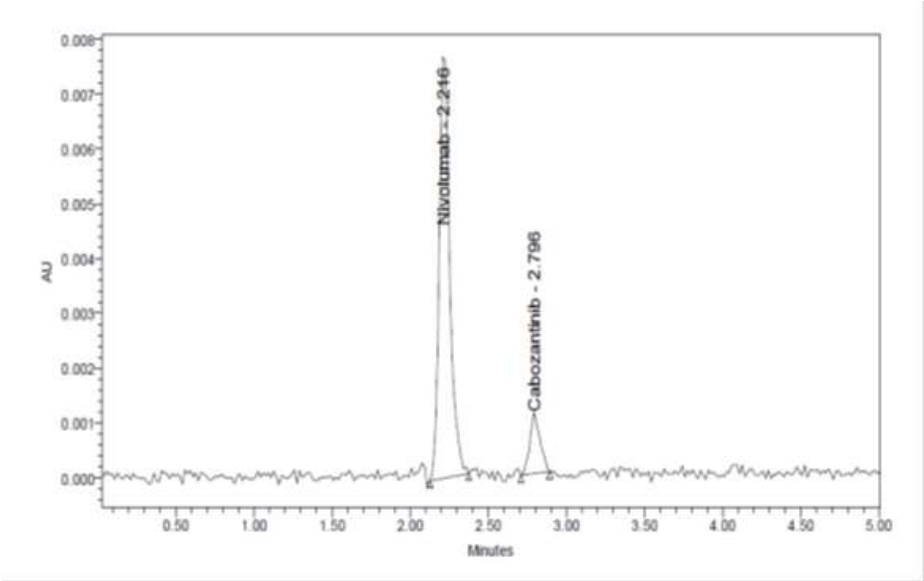


Figure 6: LOQ of NVM and CBZ

Robustness:

Robustness conditions like Flow minus (0.9mL/min), Flow plus (1.1mL/min), mobile phase minus (55B:45A), mobile phase plus (65B:35A), temperature minus (25°C) and temperature plus(35°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.

Assay:

Rhodes Pharmaceuticals 0f American Association bearing Nivolumab plus Cabozantinib Formulation bearing the Nivolumab 240mg, Cabozantinib 40mg. An assay was performed with the above formulation. The average % Assay for Nivolumab and Cabozantinib obtained was 99.66% and 99.74% respectively.

Table 5: Assay Data of Nivolumab and Cabozantinib

S.No	NVM			CBZ		
	Std. Area	Spl. Area	% Purity	Std. Area	Spl. Area	% Purity
I	1521624	1529639	100.24	234438	234138	99.47
II	1529889	1528862	100.19	238304	237068	100.71
III	1500407	1519284	99.56	233523	232840	98.92
IV	1522792	1520503	99.64	232493	237179	100.76
V	1527442	1518919	99.53	233354	233952	99.39
VI	1517329	1507599	98.79	232549	233497	99.20
Mean	1519914	1520801	99.66	234445	234779	99.74
S.D	10531.7	8041.3	0.53	2175.5	1870.7	0.8
% RSD	0.7	0.5	0.5	0.9	0.8	0.8

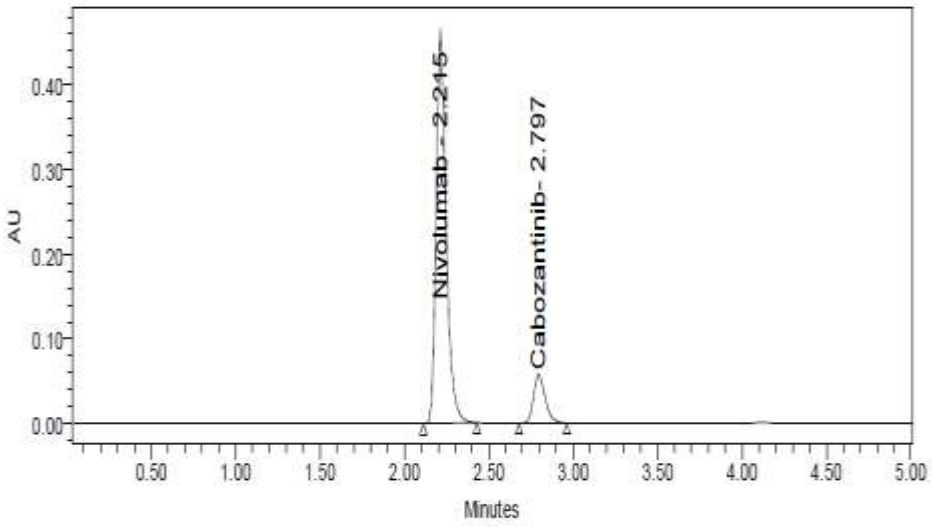


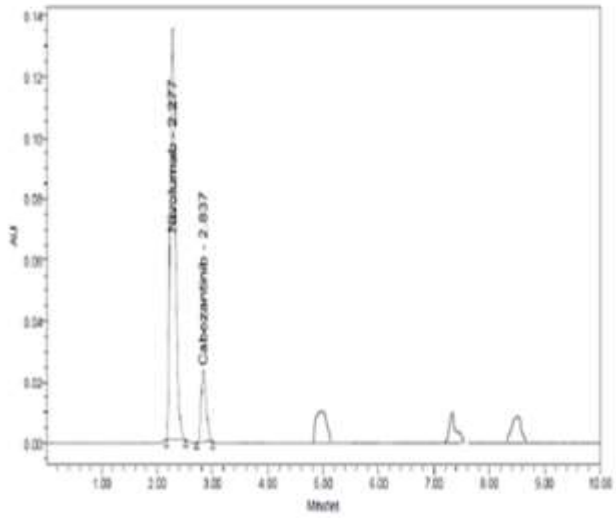
Figure 7: Chromatogram of Sample solution

Degradation studies:

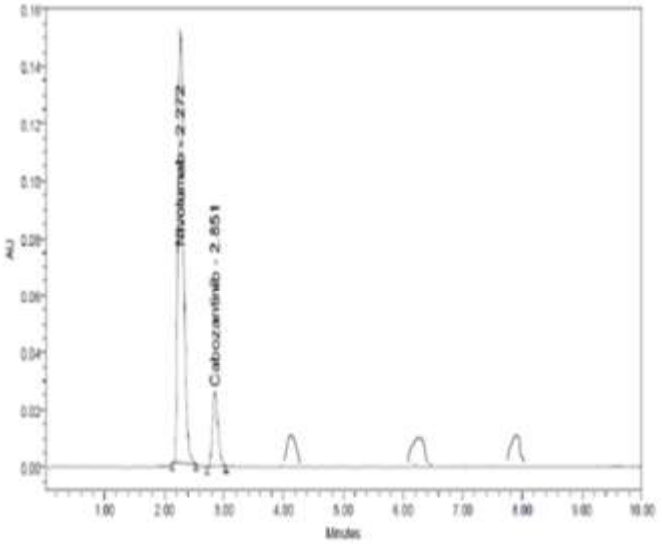
Standards and degraded samples were injected and the percentage of drug degraded in solution by applying different conditions like acid, alkali, and oxidative, photolytic, thermal, and neutral analysis. Degradation studies were performed and the obtained results were within the limits in compliance with the ICH guidelines and mass balance.

Table 6: Degradation Data for NVM and CBZ

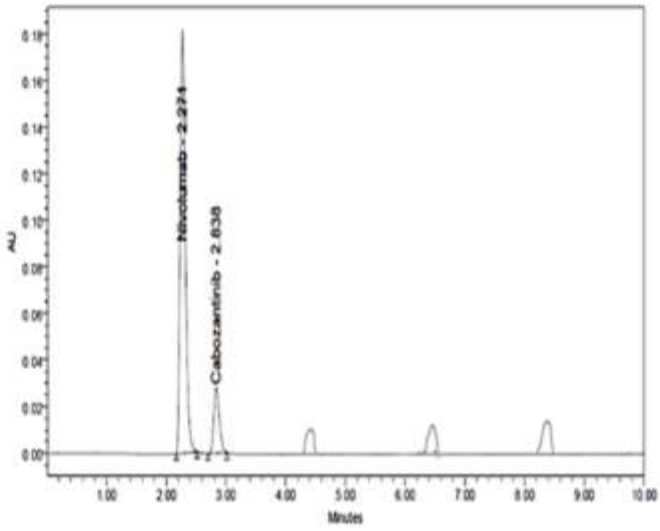
Type of Degradation	Nivolumab		Cabozantinib	
	% Assay	% Degraded	% Assay	% Degraded
Acid	92.70	7.30	93.87	6.13
Base	93.33	6.67	91.85	8.15
Peroxide	92.66	7.34	92.37	7.63
Thermal	93.44	6.56	93.20	6.80
UV	91.87	8.13	92.33	7.67
Water	92.87	7.13	91.06	8.94



Chromatogram of Acid degradation



Chromatogram of Alkali Degradation



Chromatogram of Thermal Degradation

Figure 8: Degradation data of NVM and CBZ

CONCLUSION:

A novel and sensitive method was established for the estimate of NVM and CBZ. According to ICH recommendations, this method describes the quantification of CBZ and NVM in bulk and pharmaceutical formulation as per the specific criteria. The benefit is in the ease of sample preparation and the cost-effective use of fewer chemicals. Additionally, within a period of 10 minutes, two molecules are eluted. The recommended RP-HPLC approach proved effective for accurately quantifying the compounds involved. The precision and reproducibility of the

experimental data are good, according to statistical analysis of the results. The proposed chromatographic approach is useful for routine analysis in drug research.

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