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SIMULTANEOUS METHOD VALIDATION FOR THE ESTIMATION OF NIVOLUMAB AND CABOZANTINIB IN BULK AND PHARMACEUTICAL DOSAGE FORM BY RP-HPLC METHOD

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ABSTRACT

Aim: The present study aimed to progress a modest, novel, and specific method for the simultaneous evaluation of Nivolumab and Cabozantinib in bulk and tablet dosage form by Reverse-phase high-performance liquid chromatography (RP-HPLC). **Material and Methods:** Nivolumab and Cabozantinib pure drugs (API), Combination Nivolumab and Cabozantinib Synthetic Formulation, Distilled water, Acetonitrile, Phosphate buffer, Methanol, Potassium dihydrogen ortho phosphate buffer, Ortho-phosphoric acid. All the above chemicals and solvents are from Rankem. Based on the solubility of the drugs, diluent was selected, Acetonitrile and H2O in the ratio of 50:50 v/v. **Results:** The method exhibited excellent precision with %RSD values of 0.7 for Nivolumab and 0.9 for Cabozantinib. Recovery studies revealed the method`s accuracy with recovery rates of 100.16% for Nivolumab and 99.63% for Cabozantinib. Limits of detection(LOD) and quantification(LOQ) were determined as 0.20 µg/mL, 0.03 µg/mL for Nivolumab and 0.61µg/mL,0.08 µg/mL for Cabozantinib. The regression equations obtained for Nivolumab and Cabozantinib were y =39306x + 12173 and y =34894x +1139.7 respectively. **Conclusion:** The benefit is in the ease of sample preparation and the cost-effective use of fewer chemicals and the precision and reproducibility of the experimental data are good, according to statistical analysis.

KEYWORDS: Lamivudine, Dolutegravir, RP-HPLC, Tablet dosage form, Validation, Quality control, LOD, LOQ, Recovery, %RSD.

INTRODUCTION

The quality of a drug is crucial for ensuring safety and efficacy. Quality assurance and control of pharmaceutical formulations are essential for ensuring safe and effective drugs for consumers. Analyzing pure drug substances and dosage forms is vital for their suitability in patients. The quality of analytical data depends on the methods used, so developing robust and reliable analytical methods is crucial for regulatory certification.

Drug quality and safety are ensured by controlling assay and impurities. Assay measures potency, while impurities address safety. The assay of pharmaceutical products is key to drug efficacy.

Challenges arise when developing methods for various drugs based on their properties. Achieving selectivity, speed, cost-effectiveness, simplicity, sensitivity, reproducibility, and accuracy requires researchers to find new analytical solutions for adoption in the pharmaceutical industry.

Physicochemical methods like optical, photometric, chromatographic (e.g., HPLC), and NMR/PMR are commonly used. Chemical methods include gravimetric, volumetric, and titration procedures. The growing number of new drugs demands continuous development of innovative methods to control their quality. Modern pharmaceutical analysis must meet these evolving needs.

1. The analysis should take a minimal time.
2. The accuracy of the analysis should meet the demands of Pharmacopoeia.
3. The analysis should be economical.
4. The selected method should be precise and selective

MATERIAL AND METHODS

Instruments used

An electronic balance from Denver was used for weighing purposes. The pH meter was sourced from BVK Enterprises, India. The ultrasonicator used in the experiment was also provided by BVK, India. For high-performance liquid chromatography (HPLC), a WATERS HPLC 2695 System, equipped with quaternary pumps, a photo diode, an array detector, and an auto-sample, was used. The system was integrated with Empower-2 software for analysis. For UV spectroscopy, a PG Instruments T60 UV-VIS spectrophotometer, with a special bandwidth of 2 mm and 10 mm matched quartz cells, was employed. The instrument was integrated with UV Win 6 software and was used to measure the absorbances of Nivolumab and Cabozantinib solutions.

Chemicals required: Nivolumab and Cabozantinib pure drugs (API), Combination Nivolumab and Cabozantinib Synthetic Formulation, Distilled water, Acetonitrile, Phosphate buffer, Methanol, Potassium dihydrogen ortho phosphate buffer, Ortho-phosphoric acid. All the above chemicals and solvents are from Rankem.

HPLC Method Development

Step 1 - Selection of the HPLC method and initial system. When developing an HPLC method, the first step is always to consult the literature to ascertain whether the separation has been previously performed and if so, under what conditions - this will save time doing unnecessary experimental work. When selecting an HPLC system, it must have a high probability of actually being able to analyse the sample; for example, if the sample includes polar analytes then

reverse phase HPLC would offer bothnadequate retention and resolution, whereas normal phase HPLC would be much less feasible.Consideration must be given to the following:

Sample Preparation:

- ✓ Does the sample require dissolution, filtration, extraction, preconcentration or clean up.
- ✓ Is chemical derivatization required to assist detection sensitivity or selectivity.

Types of chromatography

1. **Reverse phase** is commonly used for most samples, but for acidic/basic analytes, reverse phase ion suppression (weak acids/bases) or ion pairing (strong acids/bases) is recommended. The stationary phase should be C18 bonded.
2. For low/medium polarity analytes, normal phase HPLC is a potential candidate, particularly if the separation of isomers is required. Carbon bonded phases are easier to work with than plain silica for normal phase separations. For inorganic anion/cation analysis, ion exchange chromatography is best. Size exclusion chromatography would normally be considered for analysing high molecular weight compounds.

Column dimensions: For most samples (unless complex), long columns (25 cm) are recommended for better efficiency. Start with a flow rate of 1-1.5 ml/min. Packing particle size: 3 or 5 μm.

Detectors: Use fluorescence or electrochemical detectors for trace analysis. For preparative HPLC, use refractive index for high concentrations. Use λmax for UV detection, avoiding wavelengths <200 nm (due to noise). Higher wavelengths offer greater selectivity. Set excitation wavelength to the maximum intensity, then scan for emission intensity. System selection depends on sample/analyte nature, literature, experience, and empirical methods.

Step 2 - Selection of Initial conditions.

This step determines the optimum conditions to adequately retain all analytes; that ensures no analyte has a capacity factor of less than 0.5 (poor retention could result inpeak overlapping) and no analyte has a capacity factor greater than 10–15 (excessive retention leads to long analysis time and broad peaks with poor detectability). Selection of the following is then required:

Mobile phase solvent strength: Solvent strength measures the ability to pull analytes from the column, controlled by the concentration of the strongest solvent. In reverse phase HPLC, the organic modifier is the strong solvent; in normal phase HPLC, it's the most polar one.

Step 3 - Selectivity Optimization:

The goal is to achieve adequate selectivity (peak spacing) by optimizing mobile and stationary phase compositions. Focus on parameters likely to impact selectivity based on analyte nature. Mobile phase optimization is prioritized as it's easier and more convenient than stationary phase adjustments.

Step 4 - System parameter Optimization

This step balances resolution and analysis time after achieving selectivity. Parameters include column dimensions, packing particle size, and flow rate, which can be adjusted without affecting capacity factors or selectivity.

Step 5 - Method Validation

Proper validation of analytical methods is crucial for pharmaceutical analysis to ensure batch quality and safety. Methods should be used under GMP and GLP, following ICH guidelines (Q2A, Q2B). Key validation characteristics include accuracy, precision, specificity, detection/quantitation limits, linearity, range, robustness, and solution stability. A written and approved validation protocol is required before use.

ANALYTICAL METHOD VALIDATION

According to ICH Guidelines Method Validation can be defined as "Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics". An assay for a major component requires a different approach and acceptance criteria than a method for a trace impurity. In addition, the development of different formulations of the same drug with varying strengths or physical forms may require flexibility in method procedures.

1. System Suitability

Before analysis, system suitability tests ensure the HPLC system and method can generate accurate and precise results. Parameters such as capacity factor (K'), resolution (Rs), repeatability (RSD ≤ 2%), tailing factor (T ≤ 2), and theoretical plates (N > 2000) must meet specific criteria (Table 1.3).

2. Linearity

Linearity assesses how well the calibration plot of response versus concentration approximates a straight line. It is determined through regression analysis of data from multiple concentrations, providing a slope, intercept, and correlation coefficient.

3. Precision

Precision refers to the agreement of repeated measurements. It includes:

- **Repeatability:** Precision under the same conditions over a short period.
- **Intermediate Precision:** Precision within the same laboratory.
- **Reproducibility:** Precision between different laboratories.

4. Accuracy

Accuracy measures how close the measured value is to the true value. It is typically assessed through recovery studies, comparing results with a reference standard, spiking analytes into a blank matrix, or using standard additions.

5. Specificity/Selectivity

Specificity refers to a method's ability to respond to a single analyte, while selectivity allows for distinguishing multiple entities. A method should show no interference from extraneous components, and a representative chromatogram should confirm resolution from the analyte.

6. Robustness

Robustness is the method's capacity to remain unaffected by small, deliberate variations in parameters such as flow rate, column temperature, and mobile phase composition. Systematic variation helps assess method reliability under diverse conditions.

7. Limit of Detection (LOD)

LOD is the lowest concentration detectable but not quantifiable. It is evaluated based on visual evaluation, signal-to-noise ratio, or the standard deviation of the response. The LOD is expressed as $LOD = \frac{3.3\sigma}{S}$, where σ is the standard deviation of intercepts, and S is the mean slope of the calibration curve.

8. Limit of Quantification (LOQ)

LOQ is the lowest concentration that can be quantified with acceptable precision and accuracy. It is expressed as $LOQ = \frac{10\sigma}{S}$, with similar parameters as LOD.

Table No. 1: Characteristics to be validated in HPLC.

Characteristics	Acceptance Criteria
Accuracy/trueness	Recovery 98-102% (individual)
Precision	RSD < 2%
Repeatability	RSD < 2%
Intermediate Precision	RSD < 2%
Specificity / Selectivity	No interference
Detection Limit	S/N > 2 or 3
Quantitation Limit	S/N > 10
Linearity	Correlation coefficient $R^2 > 0.999$
Range	80 –120 %

Method preparation

Preparation of Standard stock solutions

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 make up to the final volume with diluents. (480ppm of Nivolumab and 80 ppm of Cabozantinib)

Preparation of Standard working solutions (100% solution)

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)

Preparation of Sample stock solutions

Five tablets of synthetic formulation were weighed and the average weight of each tablet was calculated, then the weight equivalent to 1tablet (240mg) was transferred into a 500mL volumetric flask, 50mL of diluents was 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)

Preparation of Sample working solutions (100% solution)

1mL of filtered sample stock solution was transferred to 10mL volumetric flask and made up with diluent. (48 ppm of Nivolumab and 8 ppm of Cabozantinib)

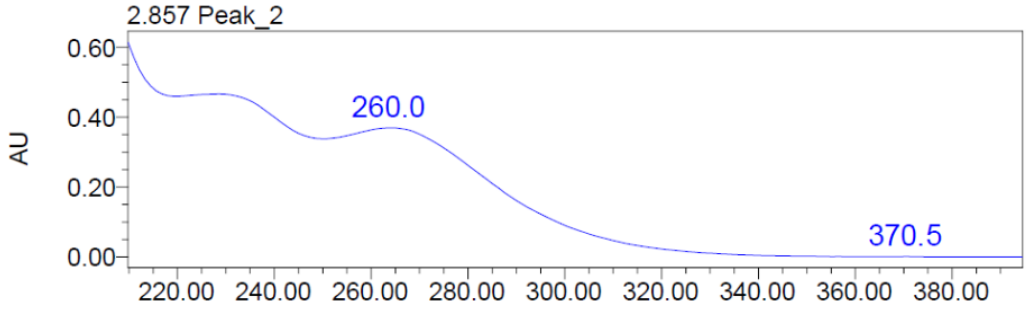
Preparation of buffer

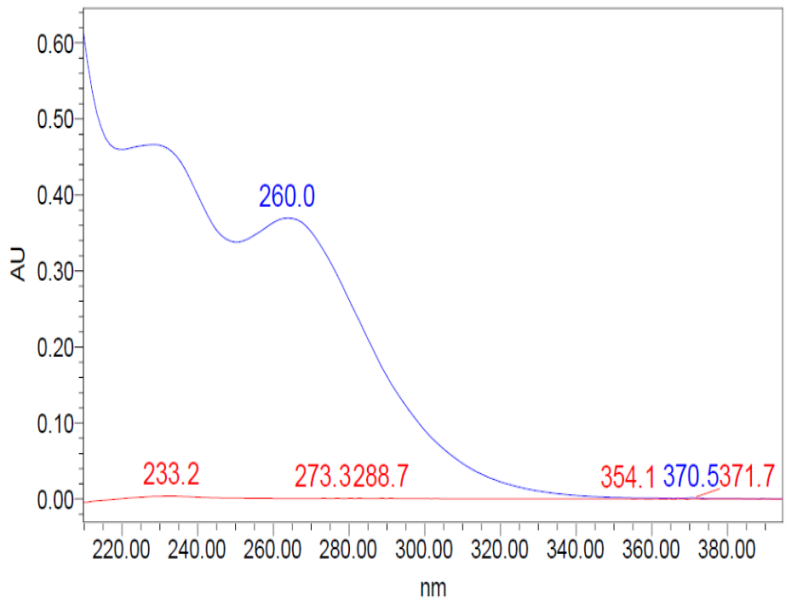
Buffer: 0.1N KH2PO4

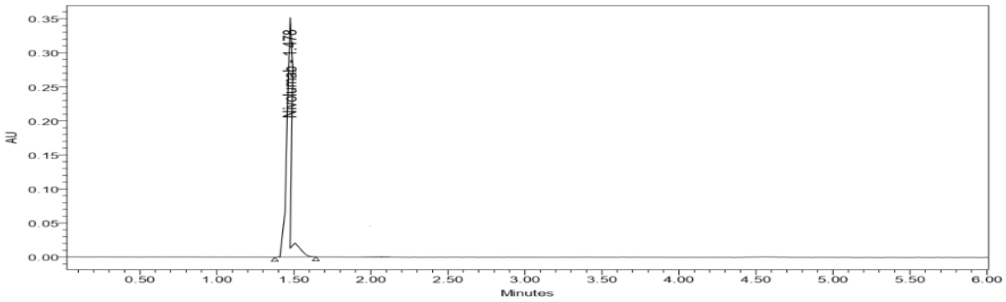
- 1.36 grams of 0.1N KH2PO4 was added to a 1000 mL VF. After adding about 900 milliliters of milli-Q water, the mixture was sonicated, allowed to evaporate, and allowed to cool. After that, water was added to make up the remaining volume, and dil.orthophosphoric acid (OPA) was used to adjust the pH to 4.8. Preparation of 0.1% OPA:
- Accurately add 1mL of OPA in a 1000mL volumetric flask, add about 900mL of milli-q water and degas to sonicate, and finally make up the volume with water.

Method Development

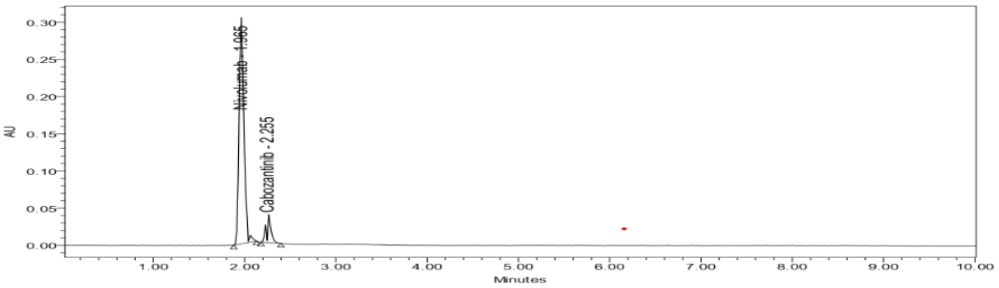
After scanning from 400 to 200 nm in UV-vis spectrophotometer, Nivolumab and Cabozantinib was showed absorption maxima at 260 nm. UV spectra of drug given in figure



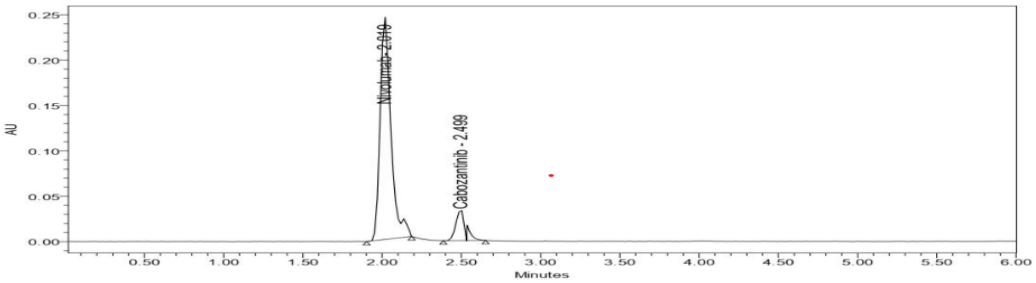




Trial – 2

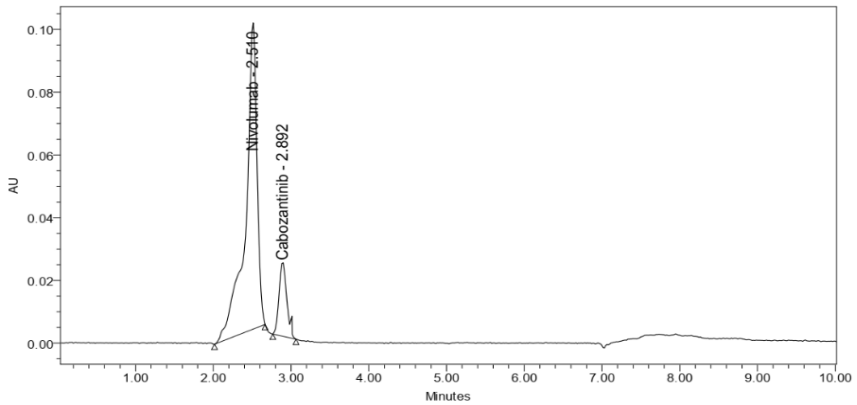


Trail – 3

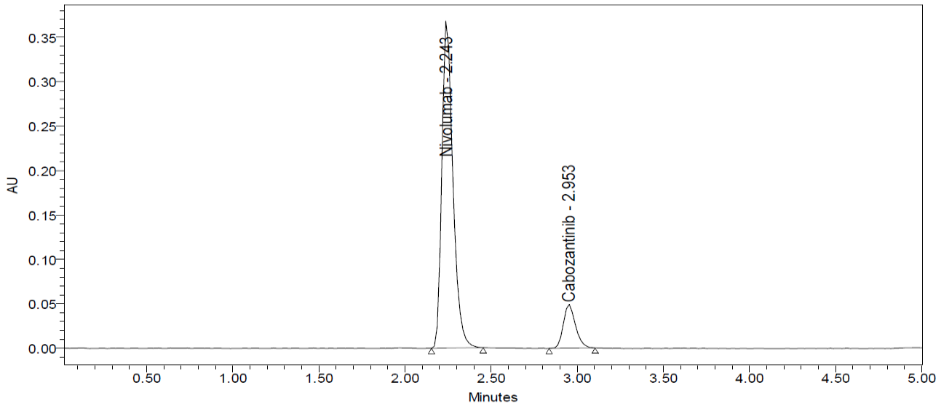


Chromatographic conditions	
Mobile phase	Water and Methanol taken in the ratio 50:50 v/v
Flow rate	1 mL/min
Column	STD Agilent C18 (4.6 x 150mm, 5µm)
Detector wavelength	260nm
Column temp	30^0c
Injection volume	10µL
Run time	6.0 min
Results	Both peaks have good resolution, tailing factor, theoretical plate count and resolution.

Trail – 4



Optimized method



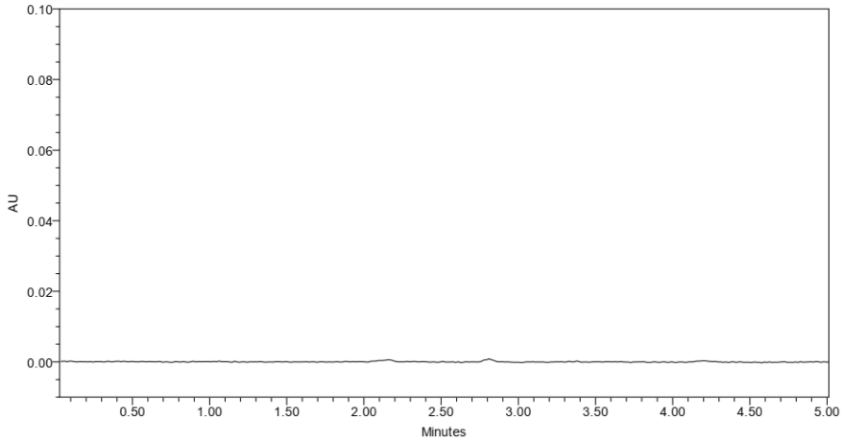
Nivolumab – 2.243

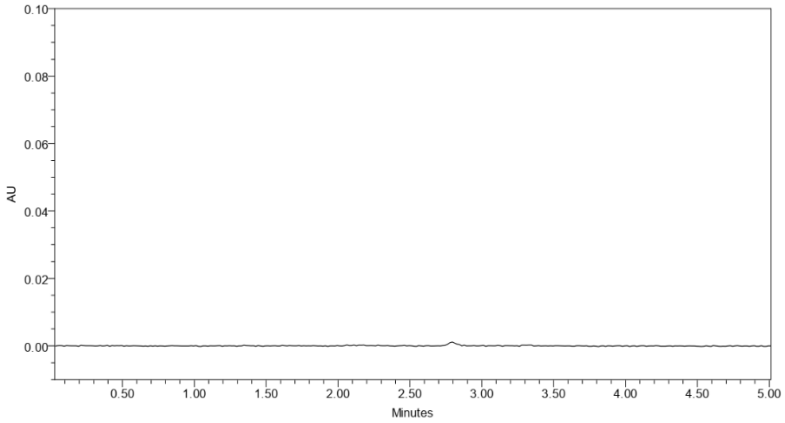
Cabozantinib – 2.953

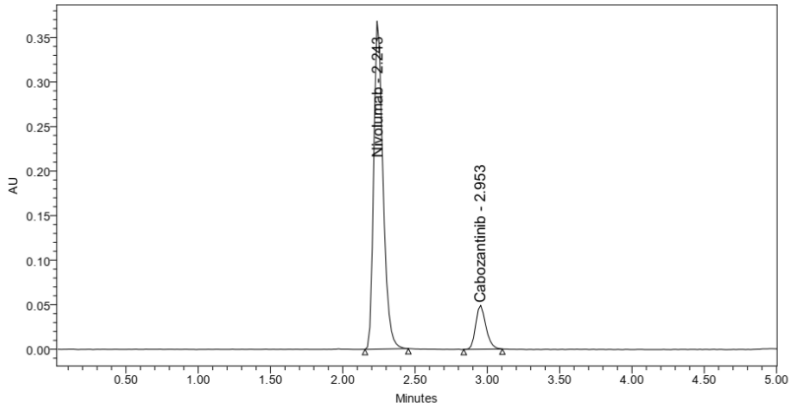
System suitability parameters

S.no	Nivolumab			Cabozantinib			
Inj	RT(min)	USP Plate Count	Tailing	RT(min)	USP Plate Count	Tailing	Resoluton
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

Method validation

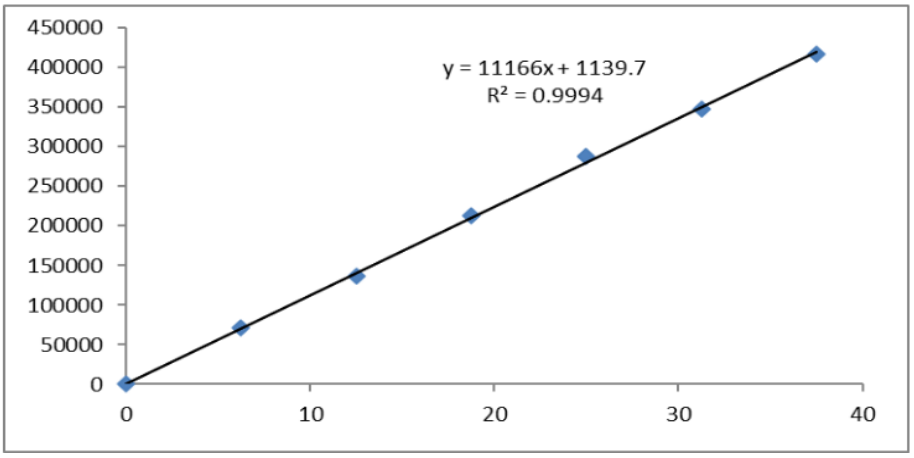


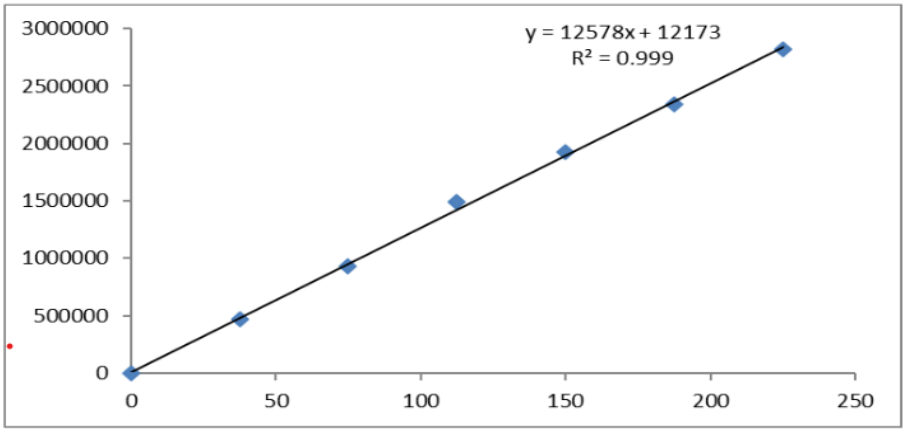




Linearity

NVM		CBZ	
Concentration (PPM)	**Peak Area (P/A)**	**Concentration (PPM)**	**Peak Area (P/A)**
37.5	473165	6.25	71631
75	934321	12.5	136918
112.5	1487481	18.75	212713
150	1926664	25	287437
187.5	2345374	31.25	347675
225	2823377	37.5	417153





Precision: From a single volumetric flask of working standard solution six injections were given and the obtained areas were mentioned above. 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 "2" the system precision was passed in this method.

Repeatability: Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated for two drugs and obtained as 0.5% andm0.8% respectively for Nivolumab and Cabozantinib. As the limit of Precision was less than "2" the system precision was passed in this method.

Accuracy: Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 100.16% and 99.63% for Nivolumab and Cabozantinib respectively.

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.

CONCLUSION

A Simple, Accurate, precise method was developed for the simultaneous estimation of the Nivolumab and Cabozantinib in Tablet dosage form. Retention time of Nivolumab and Cabozantinib were found to be 2.269 min and 2.825 min. % RSD of the Nivolumab and Cabozantinib were and found to be 0.7 and 0.9 respectively. %Recovery was obtained as 100.16% and 99.63% for Nivolumab and Cabozantinib respectively. LOD, LOQ values obtained from regression equations of Nivolumab and Cabozantinib were0.20, 0.61 and 0.03, 0.08respectively. Regression equation of Nivolumab is y = 39306x + 12173, and y = 34894x + 1139.7 of Cabozantinib. Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

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