Original Article

A New Stability Indicating Method Development and Validation Report For The Assay Of Nivolumab By RP- UPLC

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Abstract

A rapid, easy, sensitive, and selective analytical method was developed by using the reverse-phase ultra-performance liquid chromatographic technique for the assay of Nivolumab in its bulk and pharmaceutical dosage form. The elution time for the separation was 3 min ultraviolet detection was carried out at 281 nm. Efficient separation was achieved on the Waters UPLC Acquity model with an auto sampler and PDA detector. Dikma Endeversil C18 ODS (2.1x 50mm, 1.8µm) UPLC Column using the mobile phase was optimized to 0.1% of orthophosphoric acid buffer (pH 3.0): Acetonitrile in proportion 30: 70 v/v respectively as an organic solvent in a linear gradient program. Resolutions were found to be ideal by observing good peak shape and resolution at 0.25-0. 27 ml/min flow. The active pharmaceutical ingredient was extracted from tablet dosage form using a mixture of mobile phase tried was methanol: Ortho phosphoric acid buffer and Methanol: phosphate buffer, Acetonitrile: methanol with various combinations of pH as well as different proportions. The calibration graphs were linear for Nivolumab in the range of 10-50µg/ml, and the percentage recoveries for Nivolumab were found to be in the range of 99.39-100.38% respectively. The standard and sample solutions are stable for 24 hours at room temperature. A 0.45µm Nylon PTFE syringe filter can be used as an alternative to a 0.44µm syringe filter. The developed UPLC method was validated as per ICH specifications for method validation. This method can be successfully employed for the assay of Nivolumab in bulk drugs and formulations.

KEY WORDS: RP-UPLC, Nivolumab and ICH Guidelines.

INTRODUCTION:

Nivolumab is an oncologic class of drugs used to treat different types of cancers as well as tumors. [1] This includes melanoma, lung cancer, renal cell carcinoma, Hodgkin lymphoma, head and neck cancer, colon cancer, and liver cancer. It is used by slow injection into a vein, they observed side effects including tiredness, rash, liver problems, muscle pains, and cough. [2] Nivolumab is a human IgG4 monoclonal antibody that blocks the PD-1 receptor and shows the mechanism of action. It is a type of cancer treatment drug called immunotherapy and works as a turnpike inhibitor, blocking a wave that prevents activation of T cells from aggressive cancer. [3]

The first checkpoint immunotherapeutic drug to receive regulatory authorization for NSCLC is nivolumab. Nivolumab generates a partial or complete tumor response in 15-20% of patients, independent of the number of prior lines of anti-cancer therapy, by permitting host immune-mediated cytotoxic action against tumor cells. [4]

Pharmacodynamic and pharmacokinetic analysis of Nivolumab reveals that it has a PDL1-binding affinity of K_d = 2.6 nmol/l, PD1 occupancy of 85% (mean) and 72% (plateau), a half-life of 12 days for a dosage of 3 mg/kg, and 20 days for a dose of 10 mg/kg, Volume of distribution and Clearance 8.04 L, 9.50 ml/h respectively. [5]

Nivolumab quantification has been reported using a variety of analytical techniques and tools, including UV, HPLC, UPLC, LC-MS, and others. [6-11]

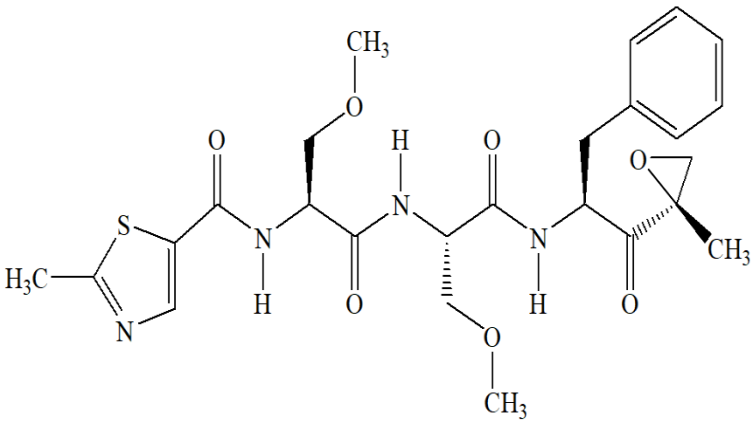

FIG 1: Chemical structure of Nivolumab

Based on the previously mentioned analytical techniques, our primary goal is to develop an efficient, rapid, sensitive, selective, linear, and accurate UPLC approach for Nivolumab determination. The process was evaluated using ICH criteria and USP 26. Linearity, accuracy, precision, specificity, the limit of detection (LOD), and the limit of quantification (LOQ) are performed and utilized to evaluate the drug concentration of Nivolumab in various pharmaceutical products by ICH Q2R1 guidelines. [12,13]

MATERIALS AND METHODS:

Chemicals and reagents

Acetonitrile, UPLC grade orthophosphoric acid, water, and sodium hydroxide were purchased from Merck (India) Ltd. Moly chem Ltd, Mumbai, India. API of Nivolumab as reference standards were procured from Glenmark and NCK pharma, Mumbai, India.

Instrumentation and chromatographic conditions

Ultra-performance liquid chromatography equipped with an auto sampler and UV detector and BEH Waters 2.1 x 50 mm, 1.7-micron UPLC Column was used for the chromatographic study. Mobile phase including a mixture of 0.1% orthophosphoric acid Buffer: Acetonitrile (30:70 v/v) was selected for the chromatographic study with a flow rate of 0.25ml/min. The auto sampler and UV detection was carried out at 281nm.

Preparation of 0.1% orthophosphoric acid buffer (pH 3)

Prepare 0.1% orthophosphoric acid buffer solution, by adding 1 ml of orthophosphoric acid in 1000 ml water and adjust the solution pH to correctly pH 3 by using sodium hydroxide.

Preparation of mobile phase

Mix a solution of 0.1% orthophosphoric acid buffer 300 ml (30%) and 700 ml Acetonitrile UPLC (70%) thoroughly

and degas in an ultrasonic water bath for 5 minutes. Filter through a 4.5 µm filter under vacuum filtration.

Diluents preparation

For the study, a diluent and sample preparation ratio of 0.1% orthophosphoric acid buffer: Acetonitrile (30:70) is selected.

Wavelength selection

UV spectrum of 10 µg/ml of Nivolumab in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. The UV spectrum wavelength was selected as 281 nm after spectrum and maximum wavelength investigation. At this wavelength, both drugs show good absorbance.

Preparation of the Nivolumab Standard and Sample Solution: (%Assay)

Standard Solution Preparation

Accurately weighed and transferred 10 mg of Nivolumab into a 10ml clean dry volumetric flask and added diluent and sonicated to dissolve it completely and make volume up to the mark with the diluent (Stock solution). Further pipette out 0.3 ml of the above stock solution into a 10 ml volumetric flask and diluted up to the mark with diluent.

Sample Solution Preparation

Accurately transferred 1 ml of Nivolumab infusion sample solution into 10 ml clean dry volumetric flask that is equivalent to 10 mg of Nivolumab drug, to that added diluent and sonicated to dissolve it completely and make volume up to the mark with the diluent (Stock solution). Further pipette out 0.3 ml of the above stock solution into a 10 ml volumetric flask and diluted up to the mark with the diluent.

Procedure

Nivolumab is available with the brand name opdyta (PD-1). Inject (six times) 5 µL of the standard, sample into the UPLC chromatographic system and measure the peak areas for the Nivolumab chromatogram and calculate the % purity.

METHOD VALIDATION:

The new analytical development method was validated as per ICH Q2 (R1) guidelines by applying various parameters like specificity, selectivity, accuracy, precision, linearity, robustness, LOD, and LOQ.

System suitability

The System suitability parameters like USP plate count, USP tailing, and resolution were measured and found to be within the limits range.

Specificity

Specificity is the ability to analyse the examiner without the presence of any other substances (impurities, spoilage products, or additives), which can be expected to be present in the samples and standards solution. It was determined by examining the solution without the sample and the samples spiked with Nivolumab.

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%). At least three injections were given at each dose and the percent recovery, % RSD was calculated.

Precision

The analytical method, in terms of precision, denotes the reproducibility of the analytical process. Six Nivolumab standard solutions were analysed and %RSD was calculated. In the brief of the procedure, six preparations containing samples were injected and % RSD and % recovery were calculated. Brief day-to-day and between-day analysis were conducted for Nivolumab.

Linearity

Linearity was obtained by preparing different values of Nivolumab at different concentration levels. Standard solutions were prepared in a concentration range of 10 ppm to 50 ppm nivolumab. Each concentration was entered into the UPLC system and the area of the peak was evaluated. Plot the graph between the peak area extracted on the Y axis and the concentration on the X axis.

Robustness

In robustness, the method was determined by making slight changes in the flow rate by 0.225 ml/min to 0.275 ml/min, organic phase by ±60-80%, and wavelength by ±10nm.

Limit of Detection and Limit of Quantification

The LOD was measured by diluting the Nivolumab standard solution and determining the concentration if the peak sample response was three times the baseline. The LOQ was measured by diluting the Nivolumab standard solution and determining the concentration if the peak response was three times the baseline noise level.

Forced degradation studies

To determine the drug substance's stability under various circumstances, such as oxidation, acidic, alkaline, thermal, and photolytic, forced degradation investigations are performed. For the oxidation degradation study, 30% hydrogen peroxide is employed. In contrast, acidic degradation is performed using 0.1N hydrochloric acid, alkaline degradation by 0.1N sodium hydroxide, photolytic degradation by UV radiation, and thermal degradation by heat. Forced degradation studies were performed by various stress conditions to obtain a degradation of about 30% breach.

RESULTS AND DISCUSSION:

Method development and optimization

Optimize the chromatographic conditions, at different concentrations levels of 0.1% orthophosphoric acid buffer and acetonitrile were tested in the mobile phase and isocratic and gradient conditions. However, the configuration of the mobile system was changed from each test to improve the resolution and achieve an acceptable retention time. Finally, 0.1% orthophosphoric acid buffer and acetonitrile in proportion 30: 70 v/v with isocratic elution were chosen because a significant response was observed in the Nivolumab solutions. During the development of the system, various stationary systems such as C8, C18 phenyl, and amino, inertsil ODS columns were tested. The Water BEH 2.1x50mm, UPLC column 1.7micron with auto sampler and UV detector is chosen for this investigation after evolution with other HPLC instruments. To achieve maximum sensitivity, the mobile phase rate and detecting wavelength were chosen to be 281 nm and 0.25 ml/min, respectively. Using the above scenario, we obtain a Nivolumab retention time of approximately 3 minutes with a pull factor of 1.34. The total signal for Nivolumab is 3599.77, % RSD for six times injection is about 0.76%; the proposed method shows that it is precise and sensitive. As per the ICH guidelines, the method was developed and validated by evaluating different parameter studies.

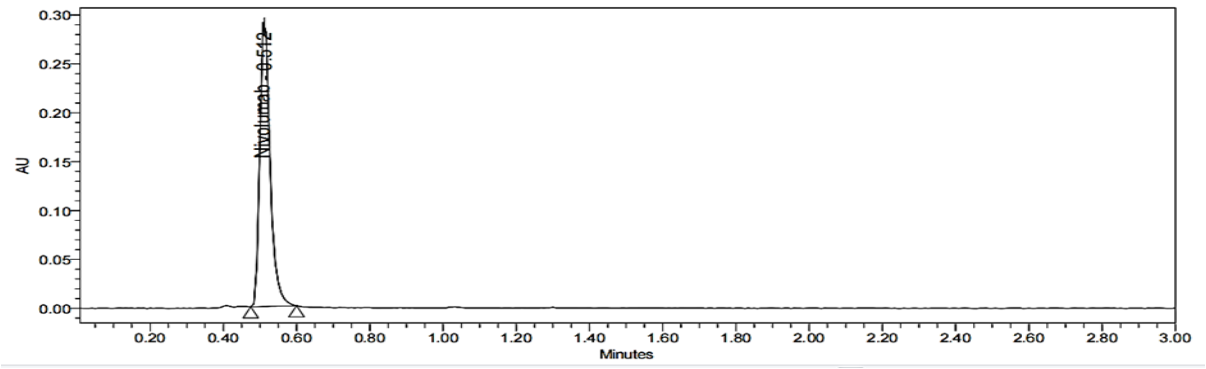

Fig 2: Chromatography of Sample

System suitability

In the system, suitability injected standard solution and reported USP tailing and plate count values are tabulated in Table 1.

Table 1: System suitability study result

System suitability parameter	Acceptance criteria	Drug name
		Nivolumab
USP Plate Count	NLT 2000	3599.77
USP Tailing	NMT 2.0	1.34
USP Resolution	NLT 2.0	2000
% RSD	NMT 2.0	0.76

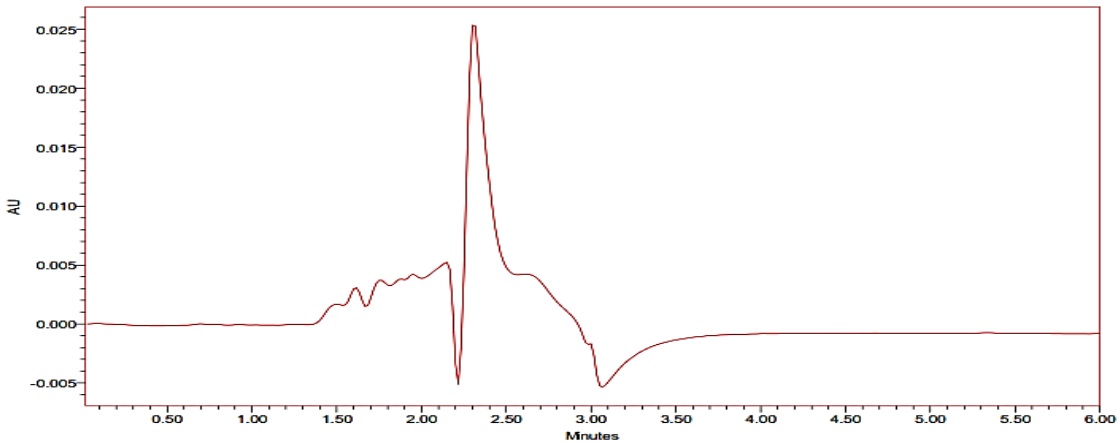

Fig 3: Chromatogram of system suitability

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.

Linearity

For the Nivolumab solution, the area of the linearity peak vs. various concentrations (µg/ml) has been calculated as 10, 20, 30, 40, and 50%, respectively. A linearity test was performed between 10 and 50 g/ml, and the correlation coefficients were higher than 0.9997(Table 2).

Table 2: Linearity Results of Nivolumab

S. No.	Concentration. µg/ml	Area count
1	10	223566
2	20	451574
3	30	686062
4	40	910009
5	50	1106177
Correlation Coefficient	0.9997	
Slope	15274	
Intercept	51822	

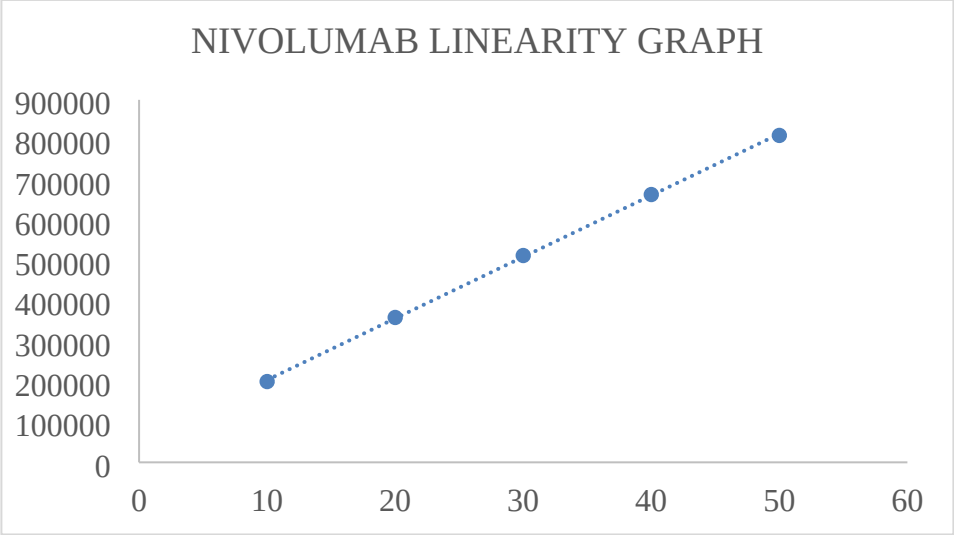

Fig 4: Calibration Plot of Nivolumab

Accuracy

In this method, accuracy was conducted in triplicate by analysing active pharma ingredient sample solution spiked with known amounts of all the impurities at three kinds of concentration levels of 50, 100, and 150% of each at a specified limit. Samples were analysed, % recoveries calculated, and it was revealed that they were within the limit. The % recovery values were found to be in the range of 99.34-100.38% for Nivolumab. The results are given in tabulated Table 3.

Table 3: Accuracy Results of Nivolumab

S. No	% Level	Nivolumab % Recovery
1	50	100.38
2	100	99.35
3	150	99.34
Mean	99.69	
SD	70.712	

Precision

Precision is the analytical method, which denotes the reproducibility of the analytical process. Precision is the degree of agreement between individual test results when a technique is subjected to several samplings of a homogeneous sample.

Intra-day precision

six replicates of a sample solution containing Nivolumab (100 µg/ml) were analysed on the same day. Peak areas were calculated, which were used to calculate mean, SD, and %RSD values. The results are represented below in Table 4.

Inter-day precision

Six replicates of a sample solution containing Nivolumab (100 µg/ml) were analysed on a different day. Peak areas were calculated which were used to calculate mean, SD, and %RSD values. The present method was found to be precise as the RSD values were less than 2% and also percentage assay values were close to 100% the results are given in Table 5

Table 4: Intra-day precision results of Nivolumab

S. No	Concentration. (µg/ml	Area counts	% Assay as is
1	100	512055	100.0
2	100	512055	100.0
3	100	516435	100.0
4	100	510956	100.0

5	100	510727	100.0
6	100	516014	100.0
%RSD		0.5	
Mean		513073.7	
SD		2494.5	

Table 5: Inter-day precision results of Nivolumab

S. No	Concentration. (µg/ml	Area counts	% Assay as is
1	100	517409	100.0
2	100	510348	100.0
3	100	513414	100.0
4	100	512149	100.0
5	100	519131	100.0
6	100	510231.1	100.0
%RSD		0.8	
Mean		519268.5	
SD		3926.6	

Limit of Detection and Limit of Quantification:

The method is evaluated according to the US FDA guidelines, and the LOD concentration for Nivolumab is 0.02, with an S/N value of 2.98. The LOQ concentration for Nivolumab is 0.07, with an S/N value of 10.

Robustness

Robustness is a measure of its capacity to stay unaffected by little, but deliberate changes in analytical process parameters indicate its consistency over time. It was performed by altering the flow rate and mobile phase ratio. Robustness results for Nivolumab were found to be within the limit and results are tabulated in Table 6.

Table 6: Robustness data of Nivolumab

Parameter Name	% RSD of Nivolumab
Flow minus (0.225 ml/min)	1.33
Actual flow (0.25 ml/min)	1.34
Flow plus (0.275 ml/min)	1.37
Organic minus (-10%)	1.50

Actual organic	1.34
organic plus (+10%)	1.20

Stability

The standard and sample solutions were kept at room temperature and at 2-8^{o} C for up to 24hrs. then the solutions were pumped into the device and calculate the % of deviation from the initial time to 24hrs. There was no significant deviation observed and confirmed that the solution was stable for up to 24hrs and % of the assay was not quite 2%. The results are given below in table 7.

Table 7: Stability results of Nivolumab

Stability	Purity	% Of deviation
24 hrs	100.0	3.94
	100.0	3.11
	100.0	4.87
	100.0	3.66
	100.0	3.11

Degradation studies

Analysis of stability testing of new substances and medicinal products the ICH guidelines are requiring, resistance testing to be performed to define the stability characteristics of active substances. The degradation results are tabulated in Table 8.

Acid degradation

In acid degradation was done at 0.1N HCl and degradation was formed to be 3.94% for Nivolumab.

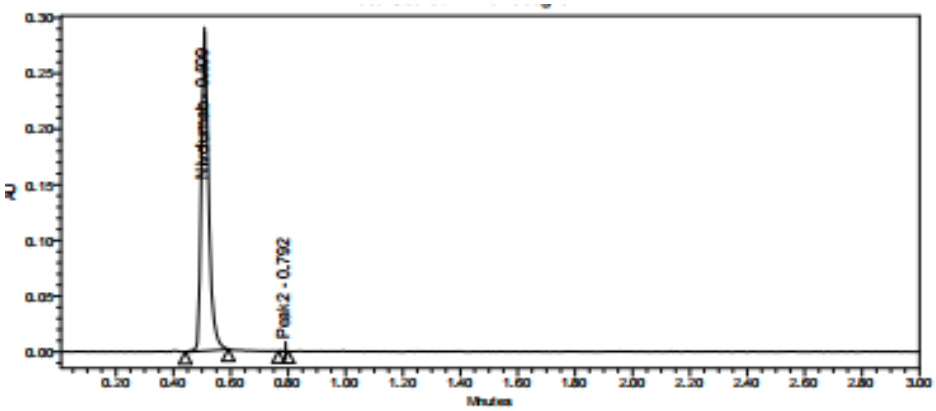

Fig 5: Chromatogram of acid degradation

Alkali degradation

In alkali, degradation was done at 0.1N NaOH, and degradation formed at 3.11% for Nivolumab (Figure 6).

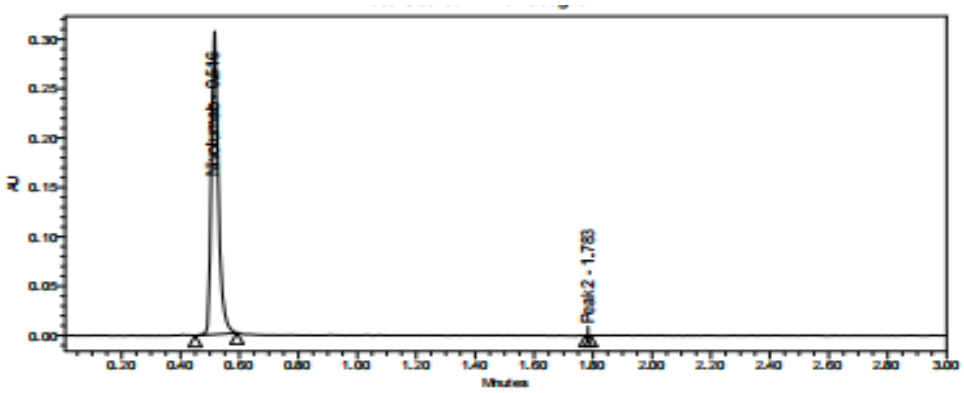

Fig 6: Chromatogram of alkali degradation

Peroxide degradation

Peroxide degradation was performed at 30% hydrogen Peroxide at 4.87% for Nivolumab (Figure 7).

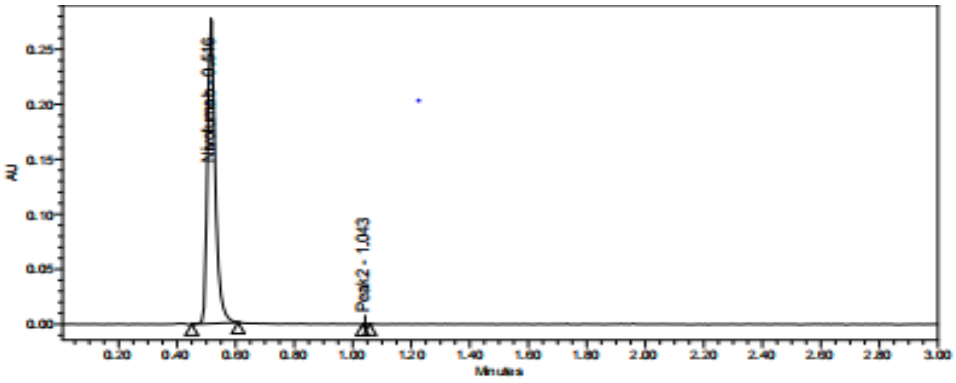

Fig 7: Chromatogram of peroxide degradation

Thermal degradation

In thermal degradation, the sample was degraded at 3.66% for Nivolumab (Figure 8).

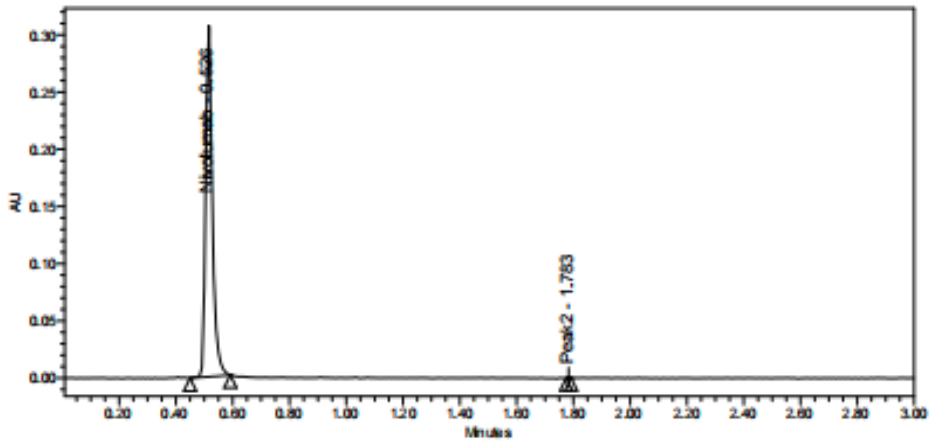

Fig 8: Chromatogram of thermal degradation

Photolytic degradation

In Photolytic s degradation, the sample was degraded at 3.11% for Nivolumab (Figure 9).

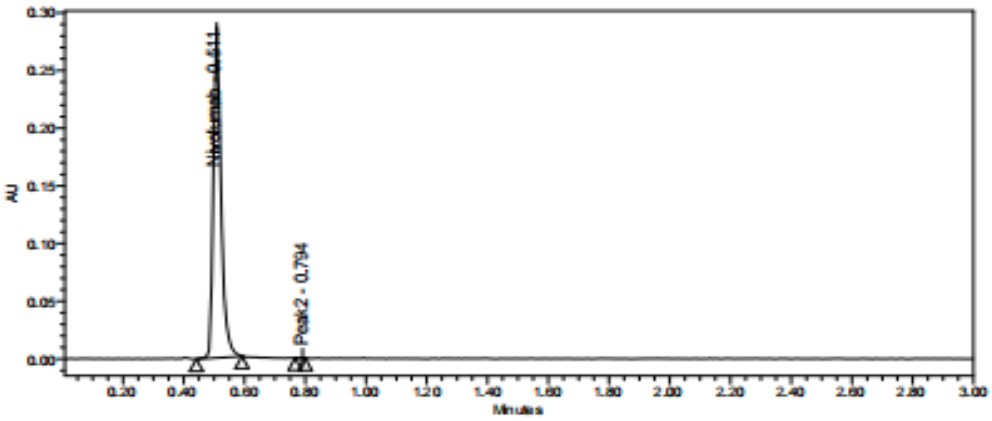

Fig 9: Chromatography of photolytic degradation

Table 8: Forced degradation results of Nivolumab

Degradation condition	Area	% Assay	% Degraded
Standard	515334	100.0	3.11
Acid degradation	515334	100.0	3.94
Alkali degradation	499327	100.0	3.11
Peroxide degradation	495047	100.0	4.87
Thermal degradation	490258	100.0	3.66
Photolytic degradation	496473	100.0	3.11

CONCLUSION

The proposed RP-UPLC system was developed and validated for the contemporaneous estimation of Nivolumab within the pharmaceutical dosage form. The proposed method was validated following ICH Q2(R1) guidelines by testing its parameters, including linearity, precision, accuracy, robustness, LOD, and LOQ. The forced degradation study proved the effectiveness of the proposed validated stability-indicating method. The newly developed method was simple, sensitive, accurate, economical, and sparing, which can be espoused in regular internal control tests in pharmaceutical industries.

Compliance and Ethical Standards

Ethical Approval: NA

Funding details: Nil.

Conflicts of interest: Declared None.

Informed consent: NA

Author's Contribution:

Each author contributed to the conception, design, and execution of the study and agreed to submit the current journal.

REFERENCE:

1. A Puszkie, G Noé, P Boudou-Rouquette, C Le-Cossec, J Arrondeau, Jean-Stephane Giraud, A Thomas-Schoemann, J Alexandre, M. Vidal, F. Goldwasser, B. Blanchet. Development and validation of an ELISA method for the quantification of nivolumab in plasma from non-small-cell lung cancer patients. J Pharm Biomed Anal. 2017; 139: 30-36.

2. G. Dionigi, V. Bianchi, F. Rovera, L. Boni, E. Piantanida, M. L. Tanda, R. Dionigi, L. Bartalena. Medullary thyroid carcinoma: surgical treatment advances. Expert Rev Anticancer Ther. 2007;7(6): 877-85.

3. B Pellegrino, A Musolino, M Tiseo. Anti-PD-1-related cryoglobulinemia during treatment with nivolumab in NSCLC patient. Ann Oncol. 2017;28(6): 1405-1406.

4. T. Tanvetyanon, B. C. Creelan, S. J Antonia. The safety and efficacy of nivolumab in advanced (metastatic) non-small cell lung cancer. Expert Rev Anticancer Ther. 2016;16(9): 903-10.

5. E. Feld, L. Horn. Emerging role of nivolumab in the management of patients with non-small-cell lung cancer: Current data and future perspectives. OncoTargets and Therapy. 2017; 10: 3697-3708.

6. A. Millet, N. Khoudour, P. Bros, D. Lebert, G. Picard, C. Machon, F. Goldwasser, B. Blanchet, J. Guitton. Quantification of nivolumab in human plasma by LC-MS/HRMS and LC-MS/MS, comparison with ELISA. Talanta. 2021; 224:121889.

7. K. Gopinath, M. Yanadirao, Y. Pavani, M.S. Rao. A Study of Method Development, Validation and Forced Degradation for Simultaneous Quantification of Cabozantinib and Nivolumab in Bulk and Pharmaceutical Dosage Form By RP-HPLC. Asian Journal of Pharmaceutical and Clinical Research. 2019;12(2): 102-106.

8. T. N.V.S.S. Satyadev. A new selective separation method development and validation of cabozantinib and nivolumab using HPLC. J. Pharm. Sci. & Res.2021;13(3): 188-192.

9. K.E. Pravallika, P. R. Auvla. Method development and validation for simultaneous estimation of Cabozantinib and Nivolumab in rat plasma by HPLC. Int. J. Pharm. Sci. Rev. Res.2020; 61(2): 8-12.

10. M. Tassi, J. De Vos, S. Chatterjee, F. Sobott, J. Bones, S. Eeltink, Advances in native high-performance liquid chromatography and intact mass spectrometry for the characterization of biopharmaceutical products. J Sep Sci. 2018;41(1):125-144.

11. D. Pluim, W. Ros, M.T.J.V. Bussel, D. Brandsma, J.H. Beijnen, J.H.M. Schellens, Enzyme linked immunosorbent assay for the quantification of nivolumab and pembrolizumab in human serum and cerebrospinal fluid. J. Pharm. Biomed. Anal. 164 (2019) 128–134 J Pharm Biomed Anal. 2019; 164:128-134.

12. United States Pharmacopeial Convention, i.e. (ed.) 2003. The United States Pharmacopeia, 26th Rev, and the National Formulary, Rockville.

13. CPMP/ICH/381/95 1994. Note for Guidance on Validation of Analytical Methods: Definitions and Terminology, Step 5.