Evaluation of a New Stability Indicating Method for the Determination of Nivolumab in Bulk and Marketed Pharmaceutical Dosage Form by Rp-Hplc

Madhuchhanda Mishra,Pragati Ranjan Satpathy, Nilima Shukla*
Department of Pharmaceutical Analysis, Sri Jayadev College of Pharmaceutical Sciences, Naharkanta, Bhubaneswar-752101
Email id: pharm.prsatpathy@gmail.com

ABSTRACT

Objective: *The current investigation was pointed at developing and progressively validating novel, simple, responsive and stable RP-HPLC method for the Quantitative Determination of Nivolumab in active pharmaceutical ingredient and Marketed Pharmaceutical Dosage form.*

Methods: *A simple, selective, validated and well-defined stability that shows isocratic RP-HPLC methodology for the quantitative determination of Nivolumab. The chromatographic strategy utilized Symmetry C18, 250 mm x 4.6 mm i.d.5µm particle size, using isocratic elution with a mobile phase consists of Methanol and Phosphate Buffer (0.02M) (pH-3.8) was taken in the ratio of 70: 30% v/v. A flow rate of 1.0 ml/min and a detector wavelength of 245nm utilizing the UV detector were given in the instrumental settings. Validation of the proposed method was carried out according to an international conference on harmonization (ICH) guidelines.*

Results: *LOD and LOQ for the active ingredients were established with respect to test concentration. The calibration charts plotted were linear with a regression coefficient of R2>0.999, means the linearity was within the limit. Recovery, specificity, linearity, accuracy, robustness, ruggedness were determined as a part of method validation and the results were found to be within the acceptable range.*

Conclusion: *The proposed method to be fast, simple, feasible and affordable in assay condition. During stability tests, it can be used for routine analysis of the selected drugs.*

Key Words: *Nivolumab, RP-HPLC, Method Development, Validation, Accuracy, Precision*.

I. INTRODUCTION

Nivolumab is a fully human IgG4 antibody targeting the immune checkpoint programmed death receptor-1 (PD-1). This antibody was produced entirely in mice and grafted onto human kappa and IgG4 Fc region with the mutation S228P for additional stability and reduced variability. It was developed by Bristol Myers Squibb. Nivolumab is a Programmed Death Receptor-1 Blocking Antibody[1]. The mechanism of action of Nivolumab is as a Programmed Death Receptor-1-directed Antibody Interaction.Nivolumab is a human monoclonal antibody to programmed cell death receptor 1 (PD-1), which acts as a checkpoint inhibitor and is used in the immunotherapy of several forms of advanced or metastatic cancer[2]. Nivolumab like other checkpoint inhibitors has major side effects and particularly immune related conditions, including acute hepatocellular and cholestatic liver injury which can be serious and even life threatening[3]. The IUPAC name of Nivolumab is Human antibody against PD-1. The Chemical Structure of Nivolumab is shown in following fig-1.

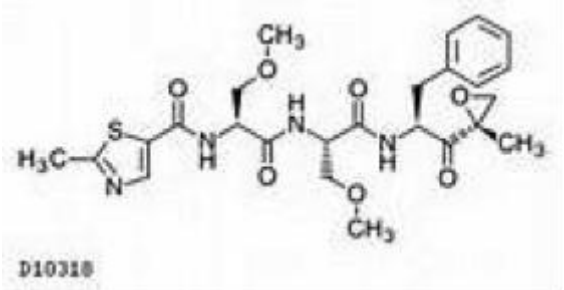

Fig-1: Chemical Structure of Nivolumab

Literature study[31-34] showed only a few analytical methods for the determination of Nivolumab in separate and combined drug dosage forms. So, we tried an attempt to develop a simple, precise and accurate method for the determination of Nivolumab in bulk and pharmaceutical dosage form by RP-HPLC.

The proposed method have considerable advantages over the existing methods, VIZ. Chromatographic method, with respect to accuracy, selectivity, sensitivity, range of determination, speed and simplicity.

II.MATERIALS AND METHODS

Materials and Instruments:

The following are the list of instruments/Equipments, chemicals/reagents and standards to perform the HPLC Analysis[4] of the drug Nivolumab.

Equipments:

Table-1: List of Equipments

S.No.	Instruments/Equipments/Apparatus
1.	HPLC WATERS with Empower2 Software with Isocratic with UV-Visible Detector.
2.	T60-LABINDIA UV – Vis spectrophotometer
3.	High Precision Electronic Balance
4.	Ultra Sonicator (Wensar wuc-2L)
5.	Thermal Oven
6.	Symmetry C_{18} Column, 250 mm x 4.6 mm and 5µm particle size
7.	P^H Analyser (ELICO)
8.	Vaccum Filtration Kit (Labindia)

Chemicals and Reagents:

Table-2: List of Chemicals used

S.No.	Name	Grade	Manufacturer/Supplier
1.	HPLC grade water	HPLC	Sd fine-Chem ltd; Mumbai
2.	Methanol	HPLC	Loba Chem; Mumbai.
3.	Ethanol	A.R.	Sd fine-Chem ltd; Mumbai
4.	Acetonitrile	HPLC	Loba Chem; Mumbai.
5.	DMSO	A.R.	Sd fine-Chem ltd; Mumbai
6.	DMF	A.R.	Sd fine-Chem ltd; Mumbai

Working Standard: Working Standard of Nivolumab: 10ppm

HPLC Instrumentation & Conditions: The HPLC system employed was **HPLC WATERS** with Empower2 Software with Isocratic with UV-Visible Detector.

Standard Preparation for UV-Spectrophotometer Analysis:

The Standard Stock Solutions– 10 mg of Nivolumab standard was transferred into 10 ml volumetric flask, dissolved & make up to volume with Methanol. Further dilutions were done by transferring 1 ml of the above solution into a 10ml volumetric flask and make up to volume with methanol to get 10ppm concentration.

It scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Nivolumab, so that the same wave number can be utilized in HPLC UV detector for estimating the Nivolumab.

Different Trials for Chromatographic Conditions:

Table-3: Different Chromatographic Conditions

Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
Develosil C_{18}, 250 mm x 4.6 mm and 5µm Column	Acetonitrile : Water = 65 : 35	0.8 ml/min	245nm	Base line noise is high	Method rejected
Develosil C_{18}, 250 mm x 4.6 mm and 5µm Column	Acetonitrile : Water = 55 : 45	0.8ml/min	245nm	Tailing is more	Method rejected

Zorbax C_{18}, 250 mm x 4.6 mm and 5µm Column	Methanol : Acetonitrile = 30 : 70	0.9 ml/min	245nm	Extra peaks	Method rejected
Phenomenex C_{18}, 250 mm x 4.6 mm and 5µm Column	Methanol : Acetonitrile = 60 : 40	1.0 ml/min	245nm	Good sharp peak	Method accepted
Symmetry C_{18}, 250 mm x 4.6 mm and 5µm Column	Methanol : Acetonitrile = 50 : 50	1.0 ml/min	245nm	Improper peak separation	Method rejected
Symmetry C_{18}, 250 mm x 4.6 mm and 5µm Column	Methanol : Phosphate Buffer (0.01M) (pH-2.8) = 40 : 60	1.0 ml/min	245nm	Tailing peaks	Method rejected
Symmetry C_{18}, 250 mm x 4.6 mm and 5µm Column	Methanol : Phosphate Buffer (0.02M) (pH-3.2) = 60 : 40	1.0 ml/min	245nm	Tailing peaks	Method rejected
Symmetry C_{18}, 250 mm x 4.6 mm and 5µm Column	Methanol : Phosphate Buffer (0.02M) (pH-3.8) = 70 : 30	1.0 ml/min	245nm	Proper Peak	Method Accepted

Preparation of 0.02M Phosphate Buffer (pH-3.8): Prepare 800 mL of distilled water in a suitable container. Add 2.72172g of Potassium dihydrogen Phosphate to the solution to the solution. Adjust solution to final desired pH 3.8 using diluted solution of orthophosphoric acid and add distilled water until volume is 1 Litre.

Preparation of Mobile Phase: Mix a mixture of 0.02M Phosphate Buffer (pH-3.8) 700 ml (70%) and 300 ml Methanol HPLC (30%) and degas in ultrasonic water bath for 15 minutes. Filter through 4.5 µ filter under vacuum filtration.

Preparation of Standard Solution:
Accurately weigh and transfer 10 mg of Nivolumab working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette out 0.1ml of Nivolumab from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Method Validation Studies: The developed analytical method was validated as per the ICH Q2 (R1) guidelines.

System Suitability: The system suitability parameters[5] like retention time, number of USP theoretical plates, USP tailing, peak area, and peak height were evaluated. A standard mixture of Nivolumabwas injected six times to determine the system suitability of the developed method.

Specificity: Specificity was determined by injecting blank and placebo samples. No peaks were observed at the retention times of Nivolumab.

Linearity and Range: Calibration curves of the three drugs were prepared at a concentration range of 6-14 µg/ml (five concentration levels) versus the peak area. The linearity was determined using the method of least square regression analysis.

Precision: The precision of an analytical method was studied by performing repeatability, intra-day, and inter-day precision as per the ICH guidelines[28-30].

Accuracy: The accuracy of the developed method was determined by calculating the recovery of the three drugs. A fixed concentration of each drug was taken (Nivolumab) was taken and the respective reference standard was added at 80%, 100%, and 120% levels. Each level was repeated three times, and the percent recovery and percent relative standard deviation were

calculated to estimate the accuracy of the developed method.

Robustness: The robustness of the developed method was studied by analyzing the effect of slight variation in the pH of mobile phase (± 0.1 units), change in flow rate (± 0.1 ml/min) and change in mobile phase composition (± 2%) on the retention time, tailing factor, theoretical plates and resolution.

Detection Limit: The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.
Quantitation Limit: The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

III. RESULTS AND DISCUSSION

Standard Preparation for UV-Spectrophotometer Analysis:

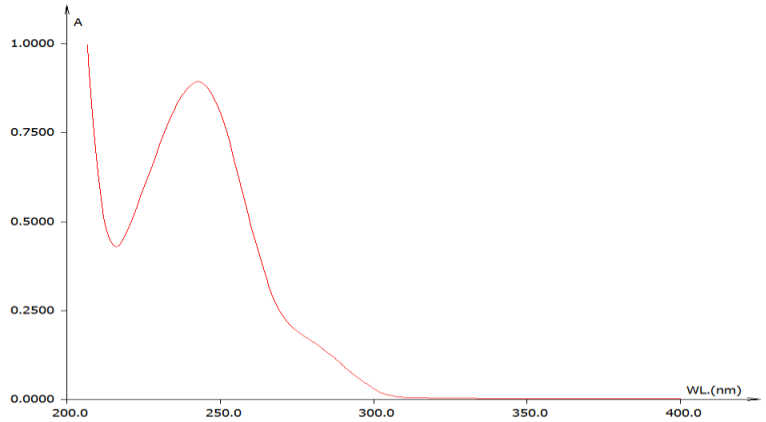

Fig-2: UV-Spectrum for Nivolumab

Observation: While scanning the Nivolumab solution we observed the maxima at 245nm.
Optimization of Analytical Method:

Optimized Chromatographic Conditions:

Column	: Symmetry C18, 250 mm x 4.6 mm i.d.5µm particle size
Mobile Phase	: Methanol: Phosphate Buffer (0.02M) (pH-3.8) (70: 30% v/v)
Flow Rate	: 1.0ml/minute
Wave length	: 245 nm
Injection volume	: 10 µl
Run time	: 7 minutes
Column temperature	: Ambient

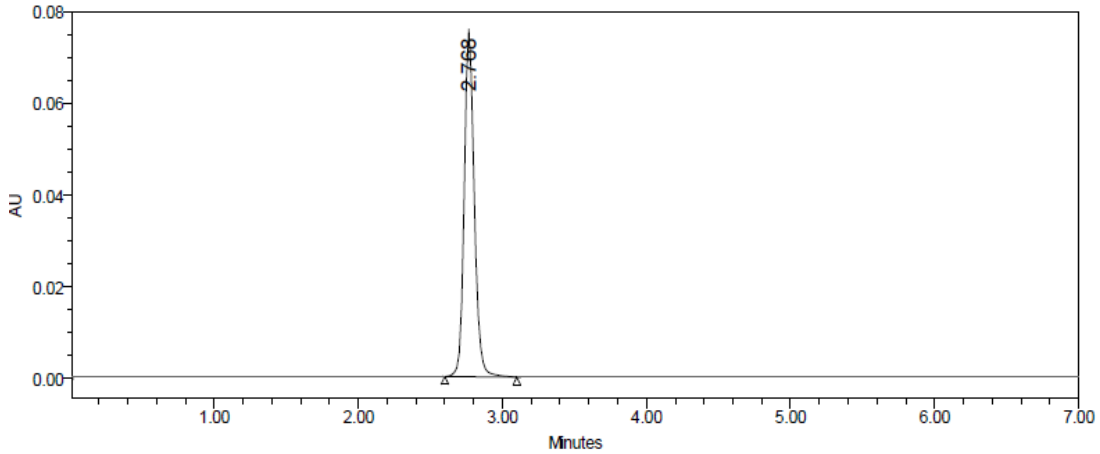

Fig-3: Optimized Chromatogram for Nivolumab

The selected and optimized mobile phase[6] was Methanol: Phosphate Buffer (70: 30% v/v) and conditions optimized were flow rate (1.0 ml/minute), wavelength (245nm), Run time was 07 mins. Here the peak has shown better theoretical plate count and symmetry. The proposed chromatographic conditions were found appropriate for the quantitative determination of the Nivolumab drug.

Analytical Method Validation

Validation of a method is the process by which a method is tested by the developer for reliability, accuracy and preciseness of its intended purpose.The proposed method isvalidated as per ICH guidelines[28-30].

System Suitability Test

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analysed constitute an integral system that can be evaluated as such. Following system suitability test parameters[7] were established. The data are shown in Table-4 & 5.

Table-4: Data of System Suitability Test

S.No.	Injection No.	RT	Area	Height	USP Plate Count	USP Tailing
1	Injection 1	2.786	715268	47844	5857	1.36
2	Injection 2	2.784	716584	46985	5986	1.38
3	Injection 3	2.768	715364	47258	5784	1.35
4	Injection 4	2.789	714895	47152	5896	1.34
5	Injection 5	2.784	716587	47258	5749	1.36
6	Injection 6	2.781	718549	47985	5657	1.39
Mean			**716207.8**		**5821.5**	**1.36**
S.D			**1347.976**			
%RSD			**0.18821**			

Table-5: Acceptance Criteria and Result:

S.No.	Parameter	Limit	Result
1	Tailing factor	$T \leq 2$	1.36
2	Theoretical plate	$N > 2000$	5821.5

Accuracy:

Recovery study:

To determine the accuracy[8] of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of Nivolumab were taken and 3 replications of each has been injected to HPLC system. From that percentage recovery values were calculated from the linearity equation y = 74143x + 7294.9. The results were shown in table-6.

Table-6: Accuracy Readings

Sample ID	Concentration (µg/ml)		Peak Area	% Recovery of Pure drug	Mean % Recovery	
	Amount Injected	Amount Recovered				
S_1 : 80 %	8	8.013	601425	100.162	Mean = 100.195%	% Mean Recovery = 100.364%
S_2 : 80 %	8	8.012	601396	100.150		
S_3 : 80 %	8	8.022	602123	100.275		
S_4 : 100 %	10	10.038	751584	100.380	Mean = 100.356	
S_5 : 100 %	10	10.039	751642	100.390		
S_6 : 100 %	10	10.030	750969	100.300		
S_7 : 120 %	12	12.057	901253	100.475	Mean = 100.541	
S_8 : 120 %	12	12.073	902431	100.608		
S_9 : 120 %	12	12.065	901864	100.541		

Observation: From the Accuracy Method, we observed that the mean %Recovery of the drug is 99.686 which are within the range of 98-102%.

Precision:

Repeatability

The precision[9] of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug Nivolumab (API). The percent relative standard deviation was calculated for Nivolumab. The results are shown in table-7.

Table-7: Results of Repeatability readings

HPLC Injection Replicates of Nivolumab	Retention Time	Peak Area	Theoretical Plates	Tailing Factor
Replicate – 1	2.777	716984	5986	1.36
Replicate – 2	2.795	715698	5897	1.37
Replicate – 3	2.789	716859	5869	1.39
Replicate – 4	2.797	718548	5967	1.37
Replicate – 5	2.797	714895	5984	1.35
Replicate – 6	2.799	715986	5879	1.38
Average		**716495**	**5930.333**	**1.37**
Standard Deviation		**1268.126**		
% RSD		**0.17699**		

Observation: From the Precision method, we observed that the %RSD of the Peak Area is 0.176 which are within the acceptable range as per ICH guidelines[10].

Intermediate Precision:

The Intermediate Precision[11] consists of two methods:-

Intra Day: In Intra Day process, the 80%, 100% and 120% concentration are injected at different intervals of time in same day.

Inter Day: In Inter Day process, the 80%, 100% and 120% concentration are injected at same intervals of time in different days.

Intra-Day:

Table-8: Peak results for Intra-Day Precision

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Nivolumab	2.784	716587	48685	1.38	5954	1
2	Nivolumab	2.768	717845	48698	1.39	5935	2
3	Nivolumab	2.786	716857	46989	1.36	5798	3
4	**Average**		**717096.3**	**48124**	**1.376**	**5895.66**	
5	**S.D**		**662.2698**				
6	**% RSD**		**0.092354**				

Inter-Day:

Table-9: Peak results for Inter-Day Precision

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Nivolumab	2.780	716987	49867	1.34	5968	1
2	Nivolumab	2.794	718695	48574	1.33	5998	2
3	Nivolumab	2.775	718542	48569	1.39	5859	3
4	**Average**		**718074.7**	**49003.33**	**1.353333**	**5941.667**	
5	**S.D**		**945.0483**				
6	**% RSD**		**0.131609**				

Observations: The intra & inter day variation of the method was carried out for standard deviation & % RSD (% RSD < 2%)

within a day & day to day variations for Nivolumabrevealed that the proposed method is precise.

Linearity & Range:

To evaluate the linearity[12] serial dilution of analyte were prepared from the stock solution was diluted with mobile phase to get a series of concentration ranging from 6-14µg/ml. The prepared solutions were sonicated. From these solutions, 10µl injections of each concentration were injected into the HPLC system and chromatographed under the optimized conditions. Calibration curve was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

Table-10: Linearity Concentrations of Nivolumab

S.No.	Concentration (in ppm)	Peak Area
1	0	0
2	6	457896
3	8	607574
4	10	752268
5	12	896587
6	14	1036579

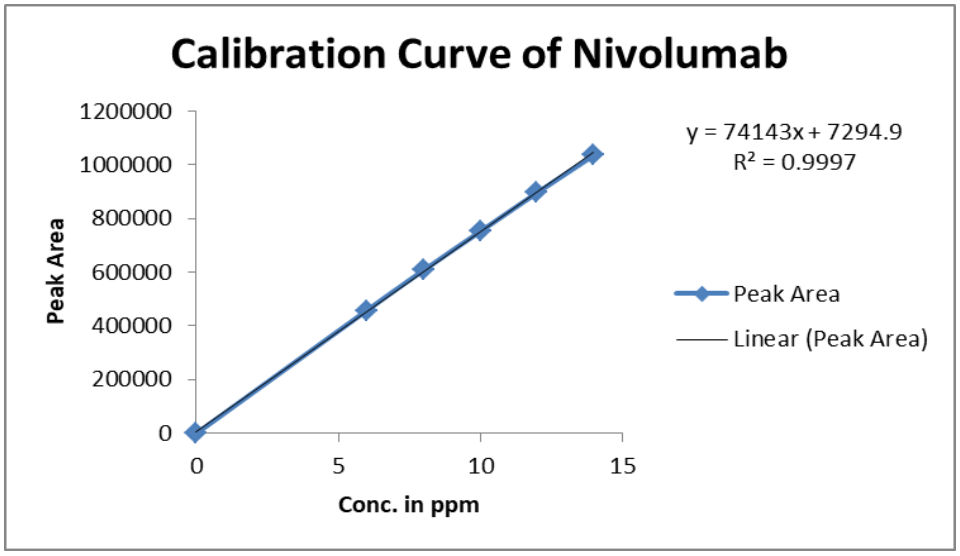

Fig-4: Calibration Curve of Nivolumab

Observation: We observed that the calibration curve showed good linearity in the range of 6-14 µg/ml, for Nivolumab with correlation coefficient (R^2) of 0.9997. A typical calibration curve[13] has the regression equation of y = 74143x + 7294.9 for Nivolumab.

Specificity: Specificity of the pharmaceutical analysis is the ability to measure accurately and specifically the concentration of API, without interference from other active ingredients, diluents, mobile phase. Solutions of mobile phase, sample solution, standard solution were injected into liquid chromatography[14]. Retention times of samples and standard were compared.

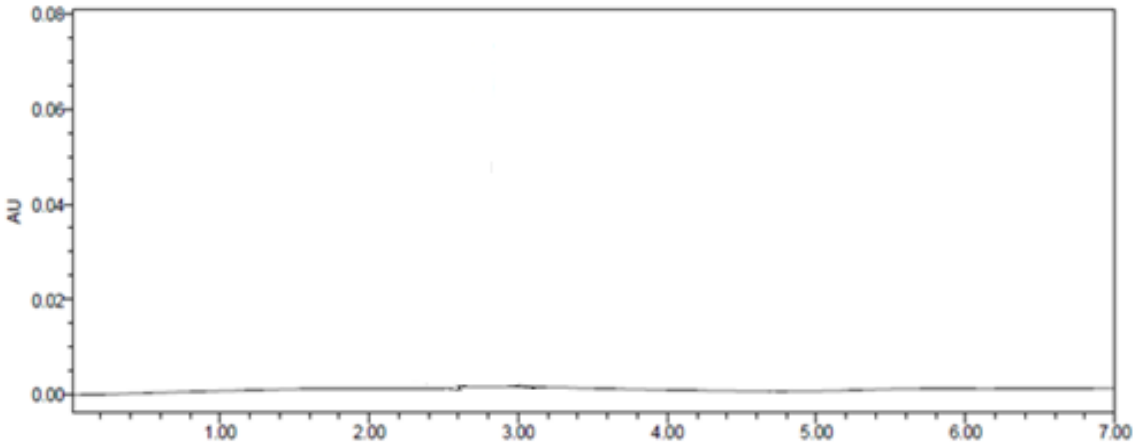

Fig-5: Chromatogram for Blank Solution

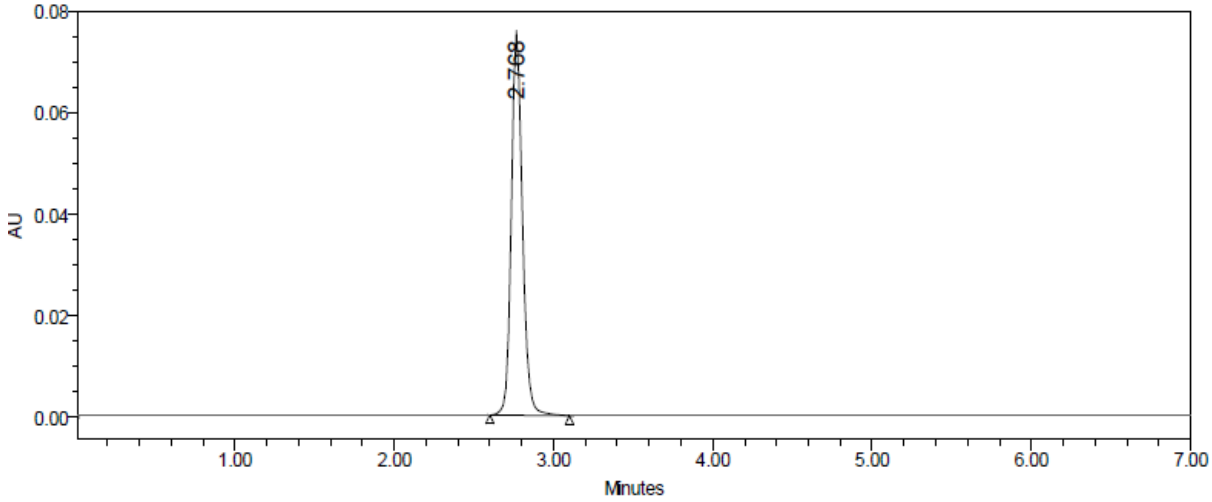

Fig-6: Optimized Chromatogram for Nivolumab Standard

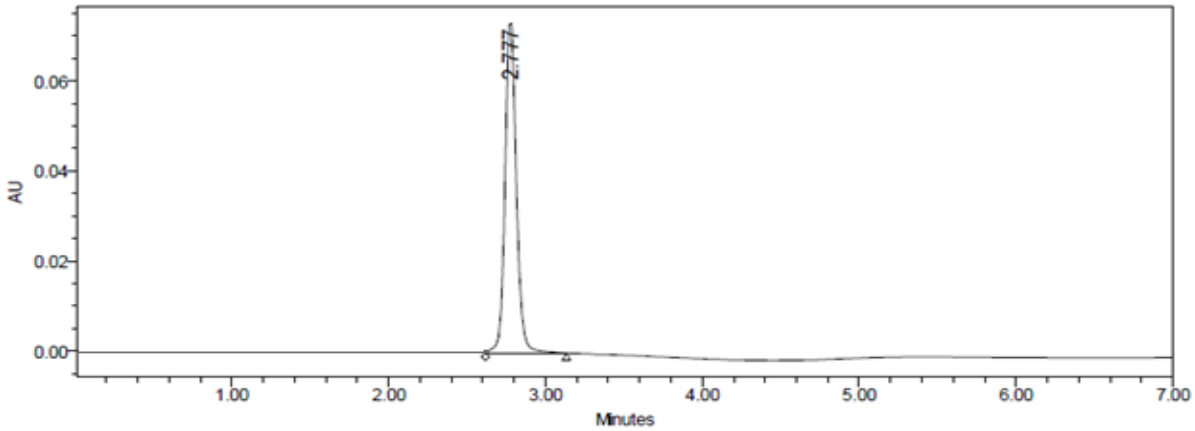

Fig-7: Optimized Chromatogram for Nivolumab Sample

Method Robustness: Influence of small changes in chromatographic conditions[15] such as change in flow rate 1ml (± 0.1ml/min), Wavelength of detection 245nm (±2nm) & organic phase content in mobile phase 60 (±5%) studied to determine the robustness[16-18]of the method are also in favour of (Table-11, % RSD <2%) the developed RP-HPLC method for the analysis of Nivolumab (API).

Table-11: Results of Method Robustness Test

Change in Parameter	Theoretical Plates	Tailing Factors
Flow (1.1 ml/min)	5954	1.35
Flow (0.8 ml/min)	6188	1.39

More Organic (70+5)	5748	1.41
Less Organic (70-5)	6185	1.48
Wavelength of Detection (250 nm)	6184	1.69
Wavelength of detection (240nm)	6247	1.47
Temperature (30 ^{0}C)	6324	1.34
Temperature (20 ^{0}C)	6985	1.32

LOD & LOQ: The detection limit[19] (LOD) and quantization limit[20] (LOQ) may be expressed as:

L.O.D. = 3.3(SD/S).

L.O.Q. = 10(SD/S)

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

The slope S may be estimated from the calibration curve[21] of the analyte.

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.507 & 1.539 µg/ml respectively.

Estimation of Nivolumab in Pharmaceutical Dosage Form

Twenty tablets were taken and the I.P. method was followed to determine the average weight. Above weighed tablets were finally powdered and triturated well. A quantity of powder equivalent to 10 mg of drug were transferred to 10 ml volumetric flask, and 8 ml of mobile phase was added and solution was sonicated for 15 minutes, there after volume was made up to 10 ml with same solvent. Then 1ml of the above solution was diluted to 10 ml with HPLC grade methanol. The solution was filtered through a membrane filter (0.45 µm) and sonicated to degas. From this stock solution (1.0 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system.

The solution prepared was injected in five replicates into the HPLC system[22] and the observations were recorded.

A duplicate injection of the standard solution was also injected into the HPLC system and the peak areas were recorded. The data are shown in Table-12.

ASSAY

% Assay=AT/AS×WS/DS×DT/WT×P/100×AW/LC×100

Where:

AT = Peak Area of Nivolumab obtained with test preparation

AS = Peak Area of Nivolumab obtained with standard preparation

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

DT = Dilution of sample solution

P = Percentage purity of working standard

Results obtained are tabulated below:

Table-12: Assay of Nivolumab

Brand Name of Tablets/Capsules	Labelled Amount of Drug (mg)	Mean (±SD) Amount (mg) Found by the Proposed Method (n=5)	Assay + % RSD
Nivolumab 40mg Injection (Oro Pharmaceuticals Pvt Ltd)	40mg	39.574 (± 0.358)	99.369% (± 0.528)

Result & Discussion: The %Purity[23]of Nivolumab 40mg Injection containing Nivolumab was found to be 99.369% (± 0.528).

Stability Studies

The results of the strain studies indicated the specificity[24]of the tactic that has been developed.Nivolumab was stable in all stress conditions[25-27] except thermal stress condition. The result of forced degradation studies are given in the following table-13.

Table-13: Results of Forced Degradation Studies of Nivolumab API

Stress Condition	Time in hrs	Assay of Active Substance	Assay of Degraded Products	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	24Hrs.	92.985	7.015	100.0
Basic Hydrolysis (0.1 M NaOH)	24Hrs.	91.062	8.938	100.0
Wet heat	24Hrs.	89.749	10.251	100.0
UV (254nm)	24Hrs.	95.625	4.375	100.0
3 % Hydrogen peroxide	24Hrs.	96.548	3.452	100.0

CONCLUSION

The analytical method was developed by studying different parameters. First of all, maximum absorbance was found to be at 245nm and the peak purity was excellent. Injection volume was selected to be 10µl which gave a good peak area. The column used for study was Symmetry C18, 250 mm x 4.6 mm i.d.5µm particle size because it was giving good peak. Ambient temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area and satisfactory retention time. Mobile phase is Methanol: Phosphate Buffer (0.02M) (pH-3.8) (70: 30% v/v) was fixed due to good symmetrical peak. So this mobile phase was used for the proposed study. Methanol was selected because of maximum extraction sonication time was fixed to be 10min at which all the drug particles were completely soluble and showed good recovery. Run time was selected to be 7min because analyze gave peak around 2.768min and also to reduce the total run time. The percent recovery was found to be 98.0-102 was linear and precise over the same range. Both system and method precision was found to be accurate and well within range. The analytical method was found linearity over the range of 6-14ppm of the Nivolumab target concentration. The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

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