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Stability Indicating Isocratic RP-HPLC Method for Simultaneous Estimation of Nivolumab and Relatlimab in Bulk and Pharmaceutical formulation

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ABSTRACT

A simple, rapid, precise, sensitive and reproducible reverse phase high performance liquid chromatography (RP-HPLC) method has been developed for the quantitative analysis of Nivolumab and Relatlimab in bulk and pharmaceutical dosage form. Chromatographic separation of Nivolumab and Relatlimab was achieved on Waters Alliance-e2695 by using Phenyl (250x 4.6mm, 5µ) column and the mobile phase containing Acetonitrile: TEA pH-2.5/OPA in the ratio of 50:50% v/v. The flow rate was 1.0 ml/min; detection was carried out by absorption at 222nm using a photodiode array detector at ambient temperature. The number of theoretical plates and tailing factor for Nivolumab and Relatlimab were NLT 2000 and should not more than 2 respectively. % Relative standard deviation of peak areas of all measurements always less than 2.0. The proposed method was validated according to ICH guidelines. The method was found to be simple, economical, suitable, precise, accurate & robust method for quantitative analysis of Nivolumab and Relatlimab.

Keywords: Nivolumab, Relatlimab, RP-HPLC, Isocratic

INTRODUCTION

Nivolumab is a monoclonal antibody that binds to the protein PD-1 on the surface of immune cells called T cells.T cells protect the body from cancer by killing certain cancer cells. But cancer cells evolve proteins to protect themselves from T cells. Nivolumab blocks those protective proteins. Thus, the T cells can kill the cancer cells.[1][2] This is an example of immune checkpoint blockade.[1][2]Nivolumab is a fully human IgG4 antibody targeting the immune checkpoint programmed death receptor-1 (PD-1).[5] 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.[5] Nivolumab is used as a first-line treatment for inoperable or metastatic melanoma in combination with Ipilimumab if the cancer does not have a mutation in BRAF,[4] and as a second-line treatment for inoperable or metastatic melanoma following treatment of Ipilimumab and, if the cancer has a BRAF mutation, a BRAF inhibitor.[5] .Nivolumab molecular formula is $C_{6362}H_{9862}N_{1712}S_{42}$ and its molar mass is 143599.39 $g.mol^{-1}$.

Relatlimab is a human IgG4 monoclonal antibody and novel immune checkpoint inhibitor that targets lymphocyte activation gene-3 (LAG-3) [3] the antagonism of which promotes T-cell proliferation, cytokine secretion, and, subsequently, restored tumor immunosurveillance.[3] It is used in combination with Nivolumab for the treatment of melanoma.[2][3]Relatilimab molecular formula is $C_{6472}H_{9922}N_{1710}N_{2024}S_{38}$ and molecular mass is 145288.79 $g{\cdot}mol^{-1}$ .Both Nivolumab and Relatlimab are immune checkpoint inhibitors. By blocking immune checkpoint proteins, which generally keep the immune responses from being too strong, immune checkpoint inhibitors restore the natural ability of T cells to attack cancer cells.

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Experimental Study

Chemicals and Reagents

NMB was procured from Shree Icon Laboratories and RMB was supplied by Sura labs, Hyderabad as gift samples. All the reagents used were of HPLC grade.

Equipment

Analysis was carried out by using Waters Alliance HPLC 2695 System fitted with quaternary pumps, Photo Diode Array Detector, and Auto Sampler integrated with Empower 2 software.

Chromatographic conditions

Mobile phase consisting of Acetonitrile:TEA pH-2.5/OPA (50:50) and it was filtered through nylon disc filter of 0.45µm (Millipore) and sonicated for 3 min before use. The flow rate was 1 mL/min and the injection volume was 10µL. PDA detection was performed at 222 nm and the separation was achieved at ambient temperature.

Preparation of standard solution

Thirty milligrams of Nivolumab and Ten mg of Relatlimb API powders were weighed and transferred into 10 mL volumetric flask, volume made with diluent to 10 mL. Further pipette out 0.4 milliliter of above solution was transferred into 10 mL volumetric flask, again volume made with diluents to obtain concentration of 120 µg/mL, and 40 µg/mL for Nivolumab and Relatlimab, respectively, said as 100% level concentrations.

Preparation of sample solution

Accurately transfer 0.1mL of Nivolumab and Relatlimab sample into a 10mL clean dry volumetric flask add diluents and sonicate it upto 30mins to dissolve, and centrifuge for 30min. to dissolve it completely and volume made with diluents to obtain concentration of 120 µg/mL, and 40 µg/mL for Nivolumab and Relatlimab respectively Prior to injecting, sample solution was filtered through 0.45 µm Nylon filter.

Method validation

System suitability test

The system suitability test of the method was carried out by injecting 100% level of working standard concentration in 6 replicates, and parameters like percentage relative standard deviation (% RSD), USP tailing factors (T), USP plate count (N), and resolution (R) were evaluated for the obtained chromatograms.

Linearity

The linearity of the method indicates that the obtained test results are directly proportional to concentration. The linearity of the proposed method has performed by injecting the series of working standard concentrations ranging from 30 µg/ml to 180 µg/ml of Nivolumab and 10 µg/mL to 60 µg/mL of Relatlimab into the HPLC system under optimized chromatographic conditions. Finally, linearity curve was constructed by taking concentration on X-axis and peak area on Y-axis and regression coefficient (R^2) value determined.

Precision

The closeness relationship among observed responses of homogenous sample on multiple replications referred as precision. Usually, it can be carried in the same day (intraday) and in different days (inter-day). Intraday and inter-day precision of the method were performed by injecting 100% level of working standard concentration for 6 times in a day and 3 times per day for three continuous days. Percentage RSD calculated for peak areas obtained

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Accuracy

The accuracy of the method was accomplished by recovery studies in which known amount samples spiked at three different standard concentration levels about 50, 100, and 150%, each level of solution injected in triplicate. The percentage mean recovery at three different levels of the drug solution was calculated.

Specificity

Specificity represents the ability of the method to assess the intended drug in the presence of other substances without interferences. Ten micro liter volume of prepared blank solution, 100% level pure working standard solution, and standard solution with placebo have been injected individually. The retention time (RT) of individual injection of standard sample solution alone and along with placebo was observed to assess any interference that has been happened with peaks of Nivolumab and Relatlimab in obtained chromatograms

Sensitivity

The LOD and LOQ were calculated by implementation of standard deviation method, in which the following formulae were used

$$LOD = 3 \times \sigma/S$$

$$LOQ = 10 \times \sigma/S$$

where σ is the standard deviation of the Y- intercept, and S is the slope of the linear curve

Robustness

The robustness of the method was checked by slightly and deliberately changing the flow rate, mobile phase composition, and maximum absorption wavelength. It can be performed by evaluating the system suitability parameters after changing the HPLC flow rate (± 0.1 mL/ min), wavelength maximum (± 2 nm), and mobile phase ratio (± 1 mL)

Forced degradation studies

In forced degradation studies, intentionally drug substance is exposed to conditions more intense than accelerated conditions. Chemical stability of the drug molecule can be depicted with forced degradation studies, which helps in successful development of stable formulation with appropriate storage conditions. ICH guidelines emphasized certain degradation conditions like acid hydrolysis, Alkali hydrolysis, oxidation, thermal degradation, and photo stability in ICH Q1A, QIB, and Q2B guidelines

Acid degradation:

Take 0.1 ml of Nivolumab and Relatlimab sample to a 10 ml vacuum flask, add app. 5 ml of diluents, followed by 1 mlof1N HCl. The vacuum flask was then maintained at 60°C for 1hour before being neutralised with 1 N NaOH and diluted to 10ml with diluent. Filter the solution using 0.22 micron syringe filters and transfer to bottles.

Alkali degradation solution

Take 0.1 ml of Nivolumab and Relatlimab sample to a 10 ml vacuum flask, add app. 5 ml of diluents, followed by 1 ml of 1N NaOH. The vacuum flask was then maintained at 60°C for 1 hour before being neutralised with 1 N HCl and diluted to 10ml with diluent. Filter the solution using 0.22 micron syringe filters and transfer to bottles.

Thermal degradation

Nivolumab and Relatlimab sample solution was taken in petridish and kept in Hot air oven at 105^0C for 3 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analysed.

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Peroxide degradation

Take 0.1 ml of Nivolumab and Relatlimab sample to a 10 ml vacuum flask, add app. 5 ml of diluents, followed by 1 ml of 3% H2O2. The vacuum flask was then maintained at 60°C for 1 hour and diluted to 10ml with diluent. Filter the solution using 0.22 micron syringe filters and transfer to bottles.

Reduction degradation

Take 0.1 ml of Nivolumab and Relatlimab sample to a 10 ml vacuum flask, add app. 5 ml of diluents, followed by 1 ml of 10% Sodium bisulphate solution. The vacuum flask was then maintained at 60°C for 1 hour and diluted to 10ml with diluent. Filter the solution using 0.22 micron syringe filters and transfer to bottles.

Photolytic degradation

Nivolumab and Relatlimab sample solution was placed in Photo stability chamber for 3 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analysed.

Hydrolysis degradation

Take 0.1 ml of Nivolumab and Relatli mab sample to a 10 ml vacuum flask, add app. 5 ml of diluents, followed by 1ml of HPL C grade water. The vacuum flask was then maintained at 60°for 1 hour and diluted to 10ml with diluent. Filter the solution using 0.22 micron syringe filters and transfer to bottles.

Assay

Assay of the method can be done by injecting subsequent injections of standard and sample solutions, both having concentration about 120 µg/mL and 40 µg/ mL of Nivolumab ,Relatlimab respectively. The preparation of standard and sample solutions was mentioned prior the in methods section.

Results

Initially, solubility studies of the both drugs were done and found that Acetonitrile: TEA pH-2.5/OPA (50:50) selected as diluent to prepare standard and sample solutions.

Method optimization

Method optimization has done by implementing trial and error method in such a way to obtain a chromatogram with good resolution (R), efficiency, accepted number of USP plates, and tailing factor. In this procedure, several trials have been done by altering mobile phase composition, columns, and flow rate. Finally, the method with Phenyl (250 × 4.6, 5 µm) column, mobile phase composition Acetonitrile: TEA pH-2.5/OPA (50:50) and a flow rate of 1.0 mL/min was selected as optimized method. The results obtained in the trial and error method, trial 7 selected as optimized conditions and optimized chromatogram shown in Fig.3.

Method validation

System suitability

Up on injecting 100% level concentration, the data obtained from chromatograms illustrated that system suitability parameters include % RSD (≤ 2), USP tailing factor (≤ 2), and USP plate count (> 2000) values shown in Table No.1 were satisfying the acceptance criteria as per Q2 specifications of ICH guidelines.

Table No.1: System suitability parameters for Nivolumab & Relatlimab

S.no	Parameter	Nivolumab	Relatlimab
1	Retention time	2.457	3.888

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2	Plate count	7651	4859
3	Tailing factor	0.95	1.06
4	Resolution	----	6.39
5	%RSD	1.03	0.14

Linearity

The linear response of the HPLC system for Nivolumab and Relatlimab was in the concentration range of 30 to 180 µg/mL and 10 to 60 µg/mL that was determined by constructing calibration curve between concentration and peak area (Table No.2, Fig.No1&2). The computed regression coefficient (R^2) value found to be 0.998 and 0.999 for Nivolumab and Relatlimab, respectively, and manifests the linearity of the method within the ICH guidelines limit

Table No.2: Results of linearity for Nivolumab &Relatlimab

S.NO	**Nivolumab**		**Relatlimab**	
	Conc.(µg/ml)	**Peak area**	**Conc.(µg/ml)**	**Peak area**
1	30.00	675471	10.00	226507
2	60.00	1305965	20.00	449123
3	90.00	2054716	30.00	679481
4	120.00	2669587	40.00	897164
5	150.00	3259845	50.00	1086755
6	180.00	4005783	60.00	1324712
Regression equation	y=22027.00x +15754.00		y=21776.00x+15130.00	
Slope	22027.00		21776.00	
Intercept	15754		15130	
R^2	0.998		0.999	

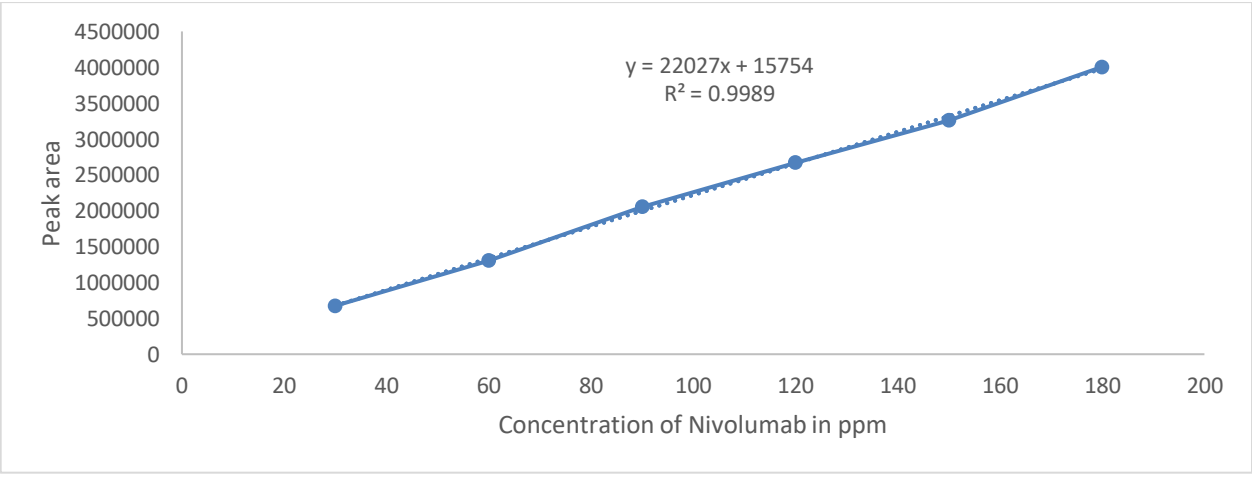

Fig 1: Linearity curve of Nivolumab

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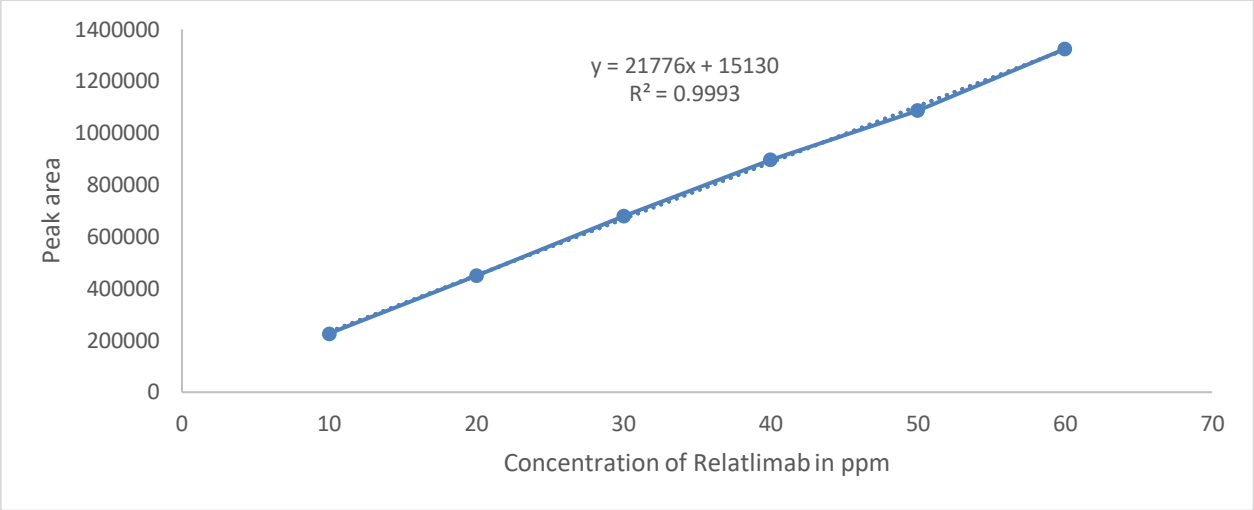

Fig 2: Linearity curve of Relatlimab

Accuracy

Percentage mean recovery of the Nivolumab and Relatlimab at three different concentration levels that were observed as 100% ± 0.1 illustrates the acceptance of the method as per Q2 specifications of ICH guidelines. Results were shown in Table No.3&4.

Table No.3: Accuracy results of Nivolumab by RP-HPLC method

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	%Recovery	Mean Recovery	%RSD
50%	1312684	0.6	0.593	98.8	99.6	1.50
	1310564	0.6	0.592	98.7		
	1345638	0.6	0.608	101.3		
100%	2666958	1.2	1.205	100.4	100.1	0.80
	2635412	1.2	1.19	99.2		
	2674582	1.2	1.208	100.7		
150%	3986574	1.8	1.801	100.1	99.9	0.49
	3995287	1.8	1.805	100.3		
	3958691	1.8	1.788	99.3		

Table No.4: Accuracy results for Relatlimab by RP-HPLC method

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	%Recovery	Mean Recovery	%RSD
50%	449152	0.2	0.201	100.5	100.0	0.50
	447633	0.2	0.2	100.0		

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	445548	0.2	0.199	99.5		
100%	896541	0.4	0.4	100.0	99.9	0.38
	891022	0.4	0.398	99.5		
	898794	0.4	0.401	100.3		
150%	1348769	0.6	0.602	100.3	100.1	0.79
	1352078	0.6	0.604	100.7		
	1331612	0.6	0.595	99.2		

Precision

Percentage RSD value of peak area responses obtained by injecting 100% level working standard solution of Nivolumab and Relatlimb were found to be 0.788 and 0.352, respectively and indicates good precision of the method.

Sensitivity

The LOD and LOQ determined as 0.36µg/mL and 1.20 µg/mL for Nivolumab and 0.12 µg/mL and 0.40 µg/mL for Relatlimb, respectively, which indicates that method has good sensitivity.

Robustness

Slightly deliberate changes in mobile phase ratio and flow rate, of the method could not produce the system suitability parameter values beyond the acceptance limits that (Table No.5&6) represent the method's robustness.

Table No.5: Robustness results of Nivolumab by RP-HPLC

Parameter	**Nivolumab**						
	Condition	**Retention time(min)**	**Peak area**	**Resolution**	**Tailing**	**Plate count**	**%RSD**
Flow rate Change (mL/min)	Less flow(0.9ml)	3.039	2510623		1.02	7718	0.31
	Actual(1ml)	2.457	2653741		1.01	7623	1.03
	More flow (1.1ml)	2.225	2840107		1.09	7845	0.45
Organic Phase change	Less Org (45:55)	2.807	2467819		1.11	7754	0.46
	Actual (50:50)	2.457	2653741		1.01	7623	1.03
	More Org (55:45)	2.065	2955346		1.09	7892	0.20

Table No.6: Robustness results of Relatlimab by RP-HPLC

Parameter	**Condition**	**Retention Time (min)**	**Peak Area**	**Resolution**	**Tailing**	**Plate Count**	**%RSD**
Flow Rate Change (mL/min)	Less flow (0.9 mL)	4.825	874,623	8.29	1.12	4,952	0.10

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	Actual (1.0 mL)	3.888	892,614	6.34	1.05	4,862	0.14
	More flow (1.1 mL)	3.008	906,243	3.16	0.99	4,763	0.25
Organic Phase Change	Less organic (63:37)	5.316	865,241	10.42	1.02	4,971	0.26
	Actual (70:30)	3.888	892,614	6.34	1.05	4,862	0.14
	More organic (77:23)	3.253	915,487	4.37	0.95	4,719	0.15

Forced degradation

In general, the acceptable percentage of degradation in a stability indicating method is not more than 20%. Percentage degradation was calculated by comparing the peak areas of 100% level working standard concentration at normal and stress conditions. Results were shown in Table No.7 and in Figures (No. 4 to 27).

TableNo.7: Forced Degradation results for Nivolumab and Relatlimab

Results: % Degradation results	**Nivolumab**					**Relatlimab**				
	Peak Area	**% Assay**	**% Deg**	**Purity Angle**	**Purity Threshold**	**Peak Area**	**% Assay**	**% Deg**	**Purity Angle**	**Purity Threshold**
Control	2653487	100	0	1.058	7.345	895746	100	0	0.676	3.954
Acid	2293008	86.4	13.6	1.062	7.362	795478	88.8	11.2	0.654	3.936
Alkali	2311479	87.1	12.9	1.054	7.327	788514	88.0	12.0	0.617	3.951
Peroxide	2254103	84.9	15.1	1.004	7.352	777596	86.8	13.2	0.637	3.974
Reduction	2604722	98.1	1.9	1.026	7.339	871325	97.2	2.8	0.661	3.928
Thermal	2384704	89.9	10.1	1.078	7.321	875402	97.7	2.3	0.641	3.935
Photolytic	2581987	97.3	2.7	1.083	7.368	882461	98.5	1.5	0.674	3.949
Hydrolysis	2619521	98.7	1.3	1.055	7.343	888238	99.1	0.9	0.682	3.905

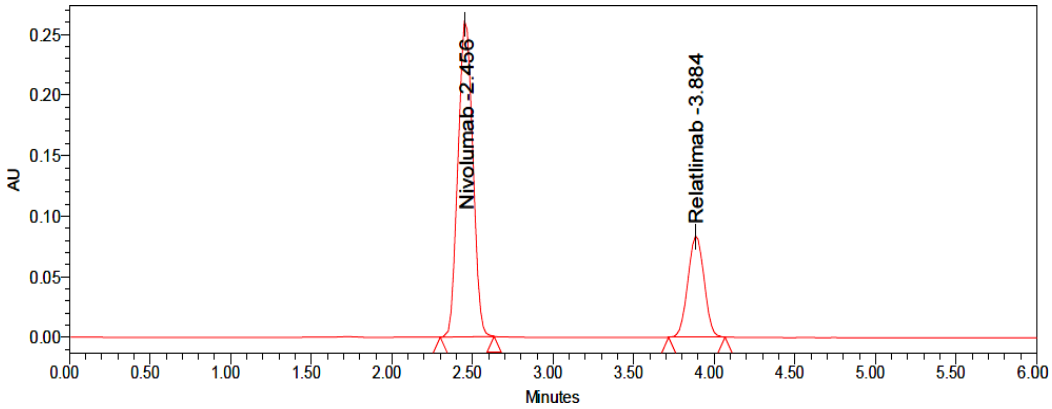

Fig .3: Optimized chromatogram of Nivolumab and Relatlimab

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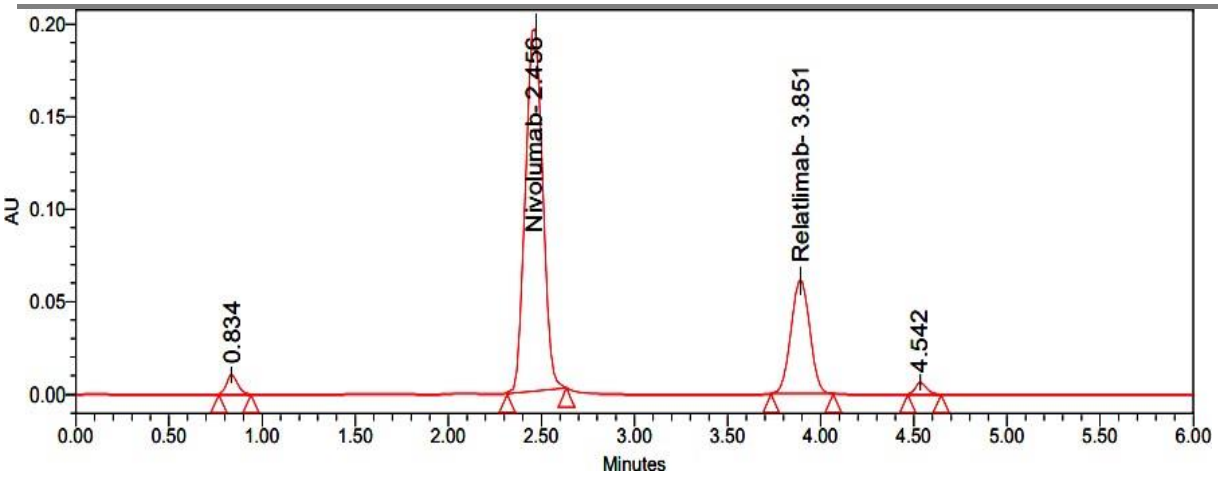

Fig. 4 : Chromatogram of Acid Degradation

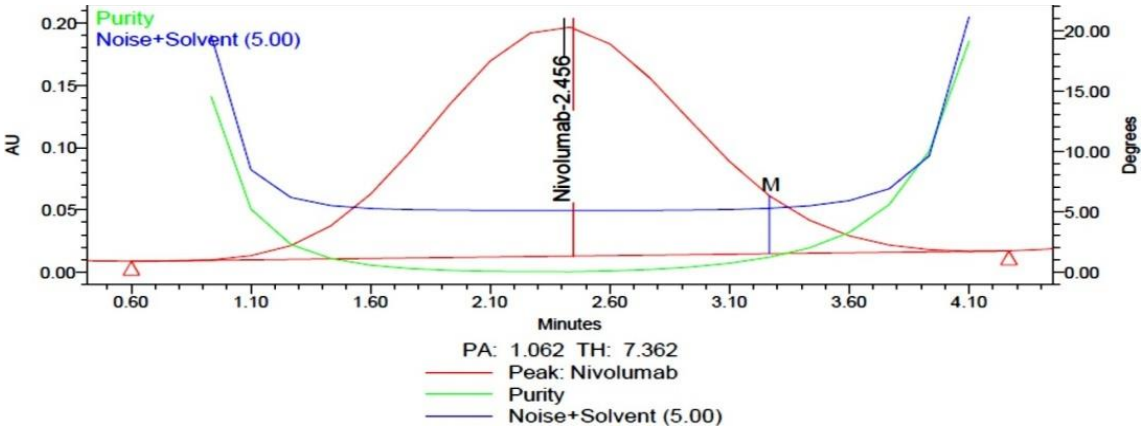

Fig.5: Purity Plot of Nivolumab

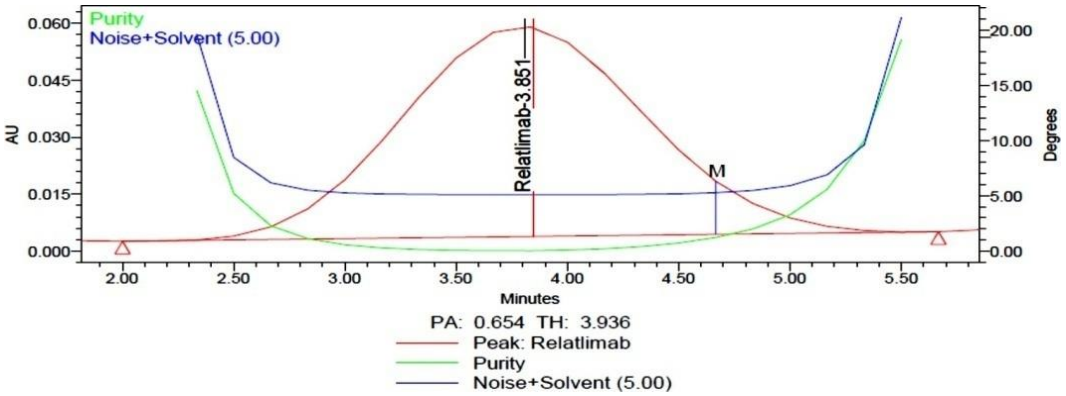

Fig.6:Purity Plot of Relatlimab

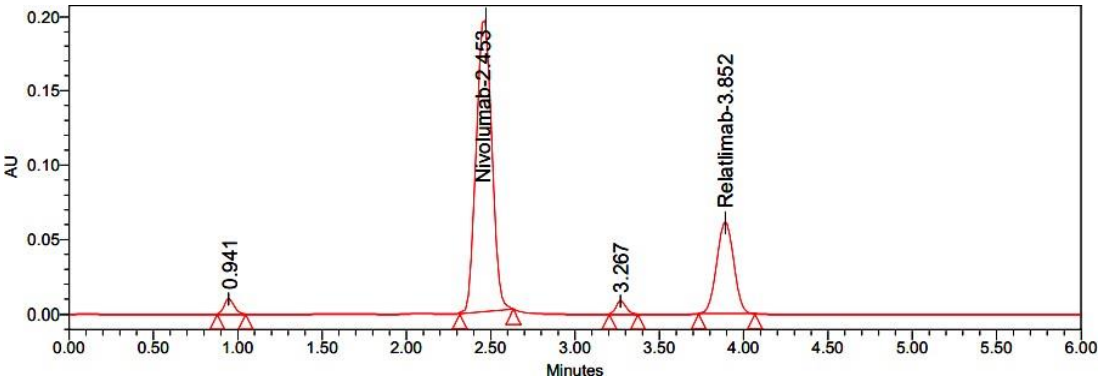

Fig.7: Chromatogram of Alkaline Degradation

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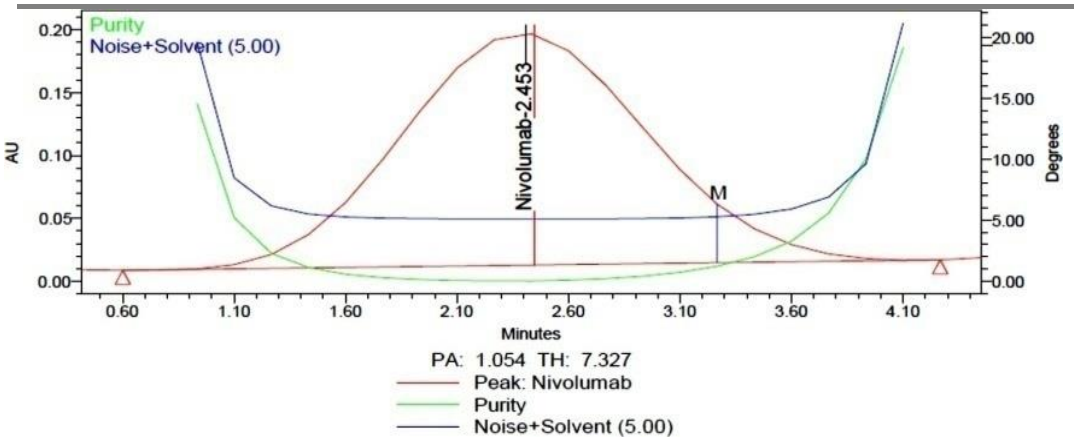

Fig.8: Purity Plot of Nivolumab

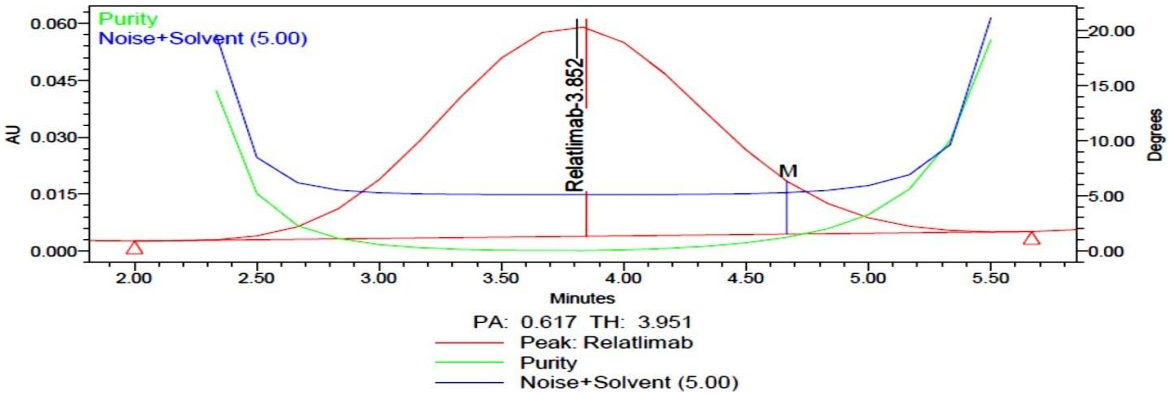

Fig.9: Purity Plot of Relatlimab

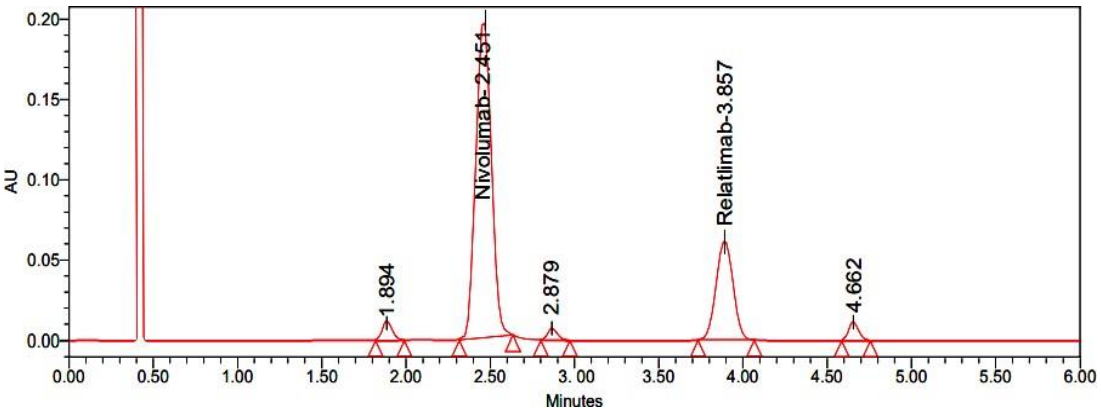

Fig.10: Chromatogram of Peroxide Degradation

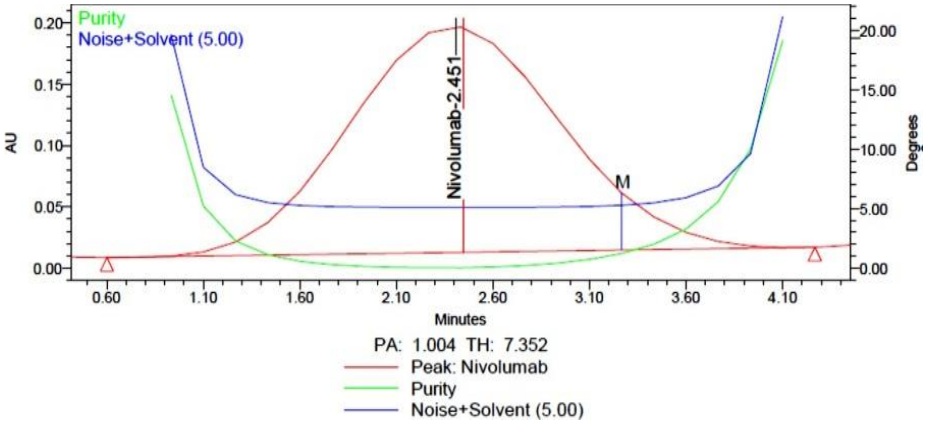

Fig.11: Purity Plot of Nivolumab

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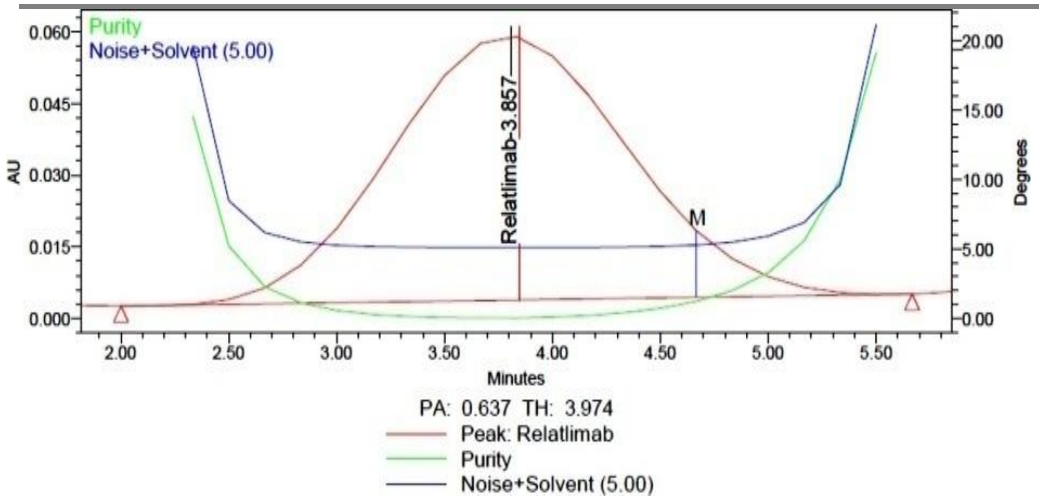

Fig.12: Purity Plot of Relatlimab

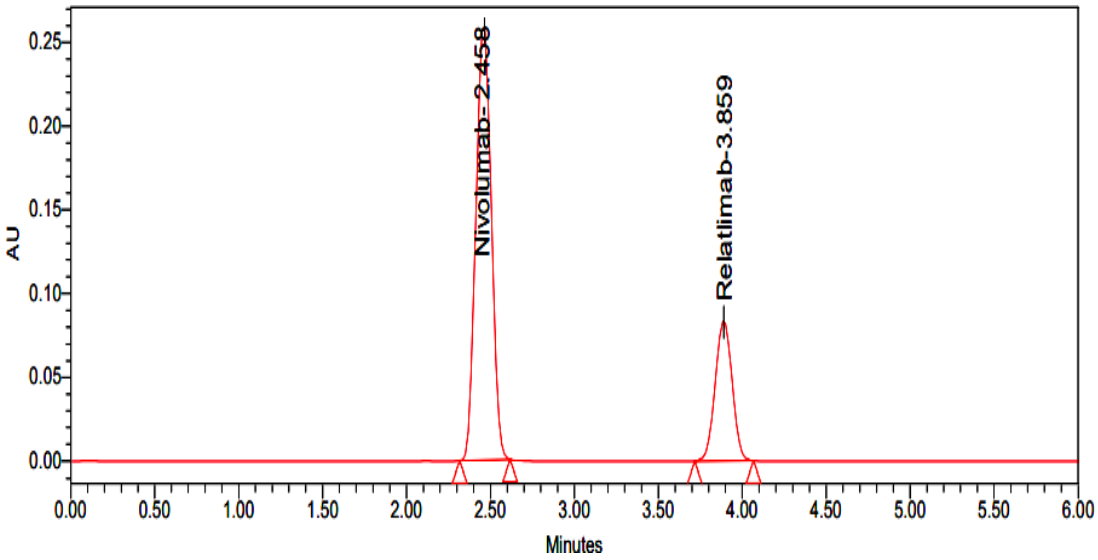

Fig.13: Chromatogram of Reduction Degradation

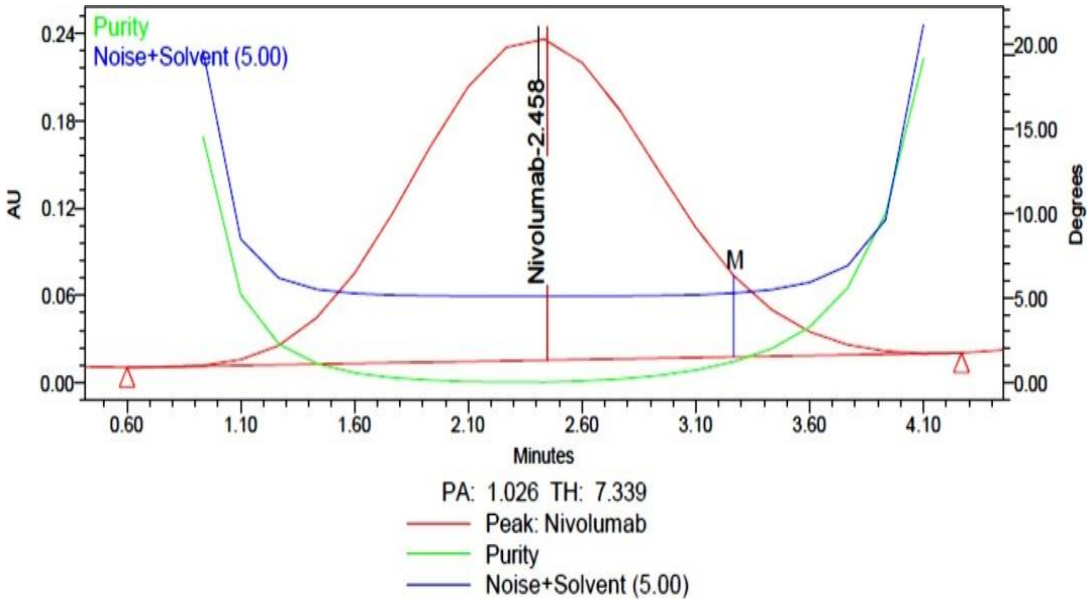

Fig.14: Purity Plot of Nivolumab

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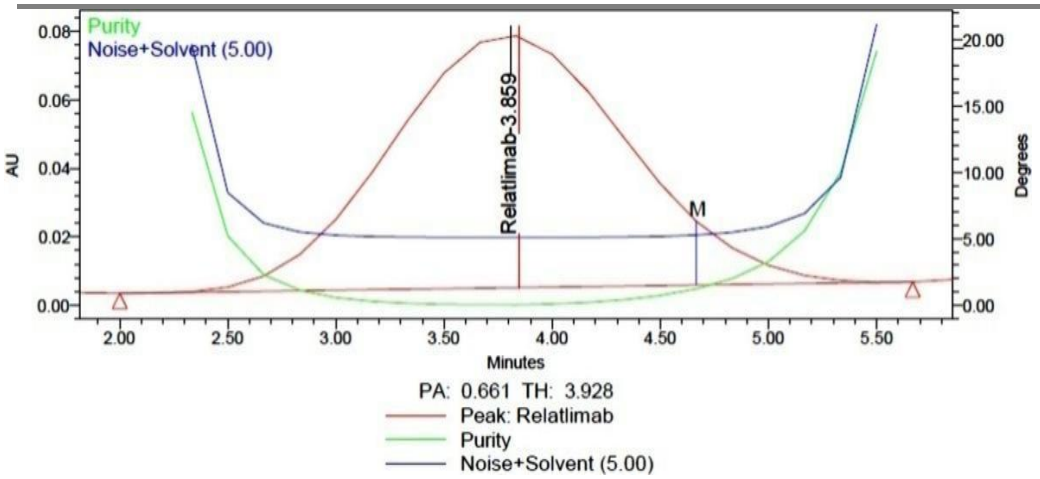

Fig.15: Purity Plot of Relatlimab

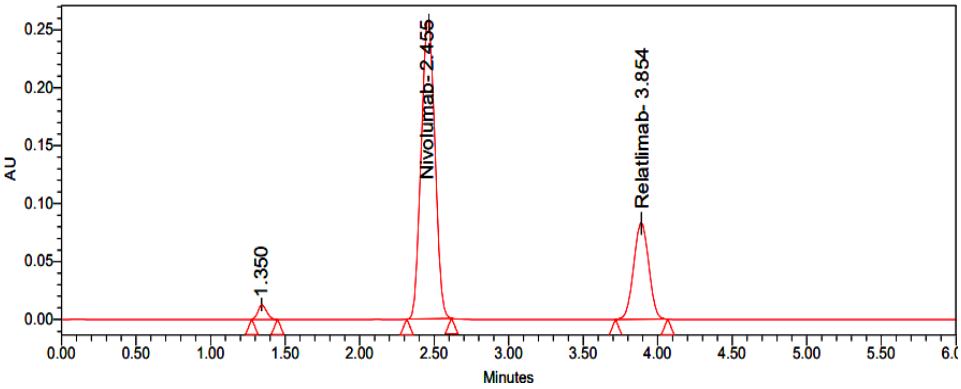

Fig.16: Chromatogram of Thermal Degradation

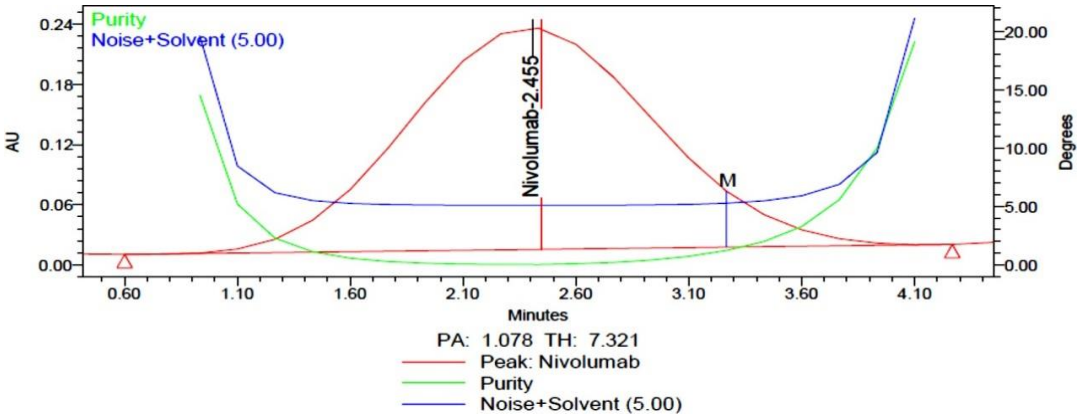

Fig.17: Purity Plot of Nivolumab

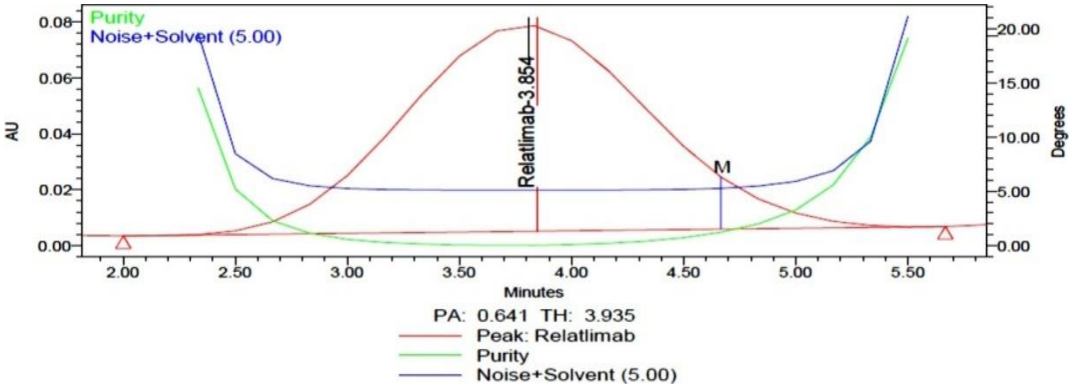

Fig.18: Purity Plot of Relatlimab

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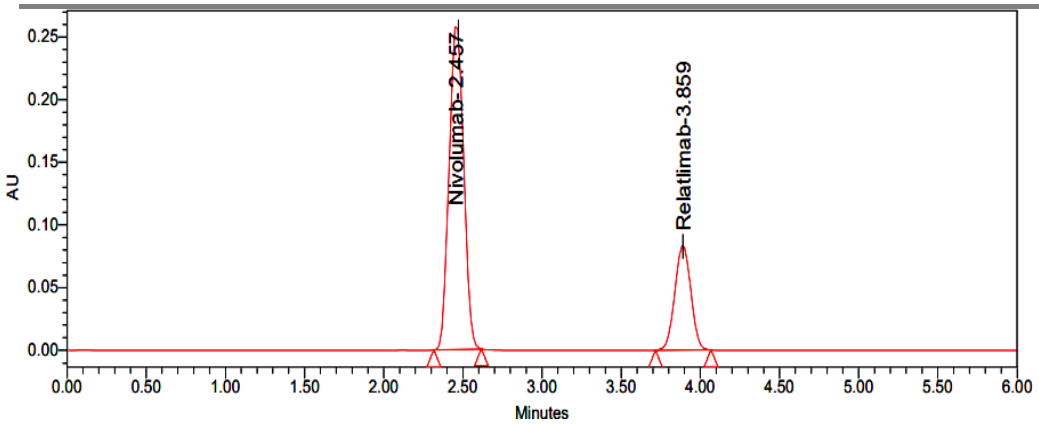

Fig.19: Chromatogram of Photolytic Degradation

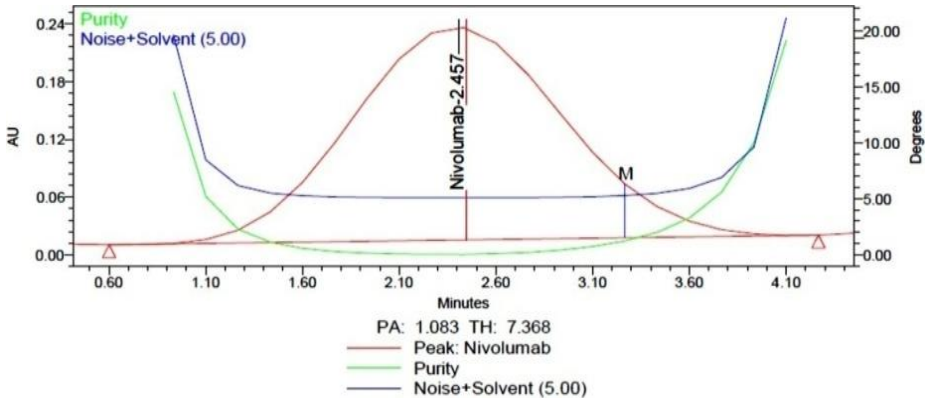

Fig .20: Purity Plot of Nivolumab

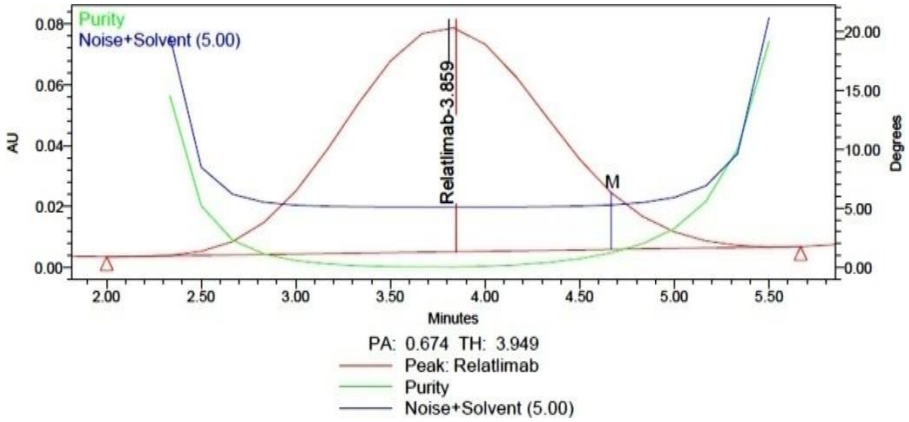

Fig.21: Purity Plot of Relatlimab

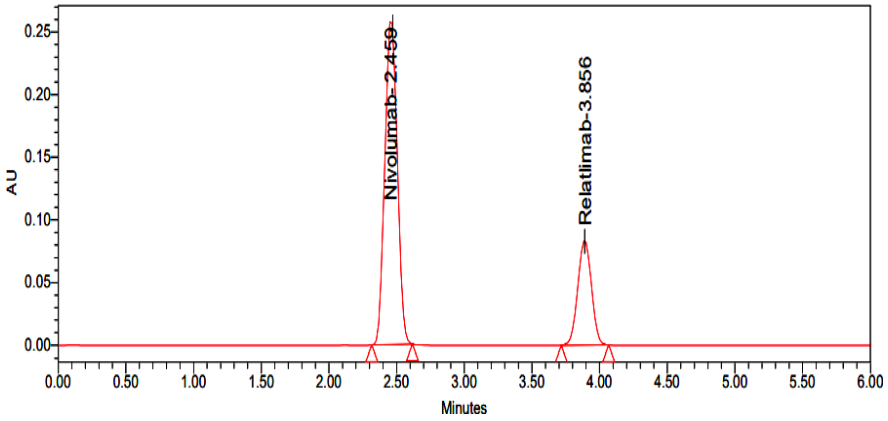

Fig.22: Chromatogram of Hydrolysis Degradation

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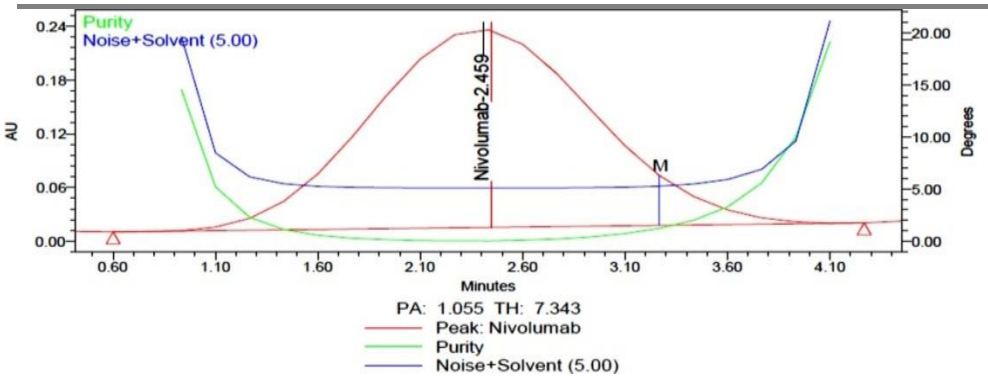

Fig.23: Purity Plot of Nivolumab

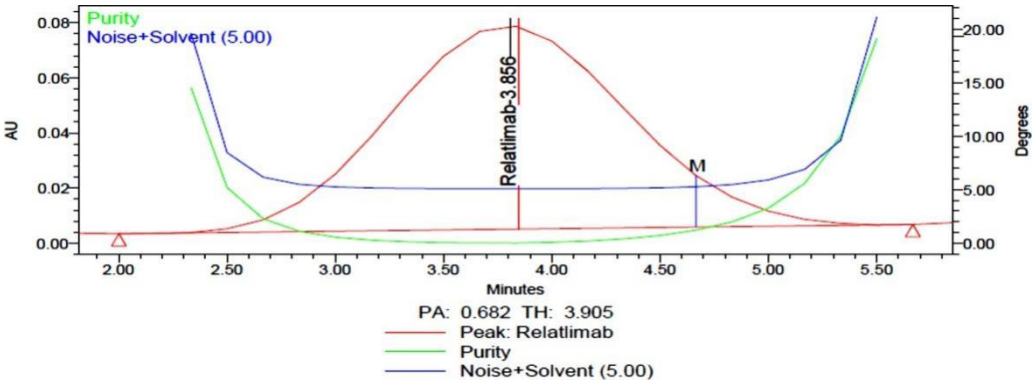

Fig.24: Purity Plot of Relatlimab

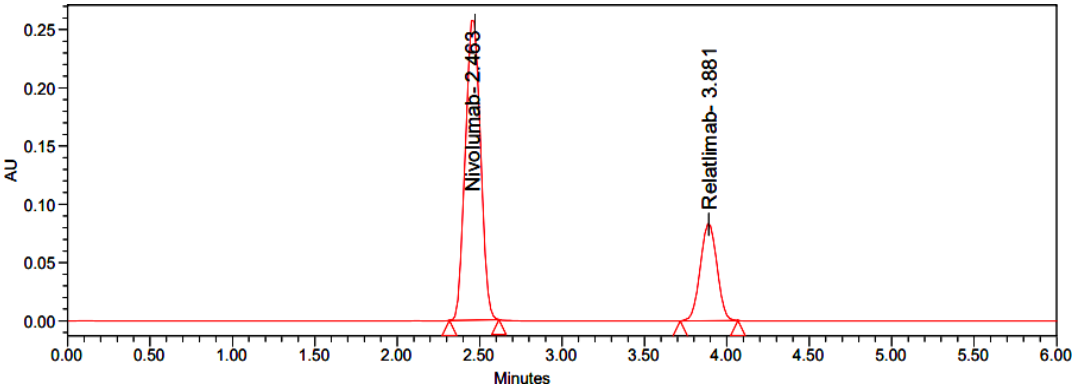

Fig.25: Chromatogram of Control Degradation

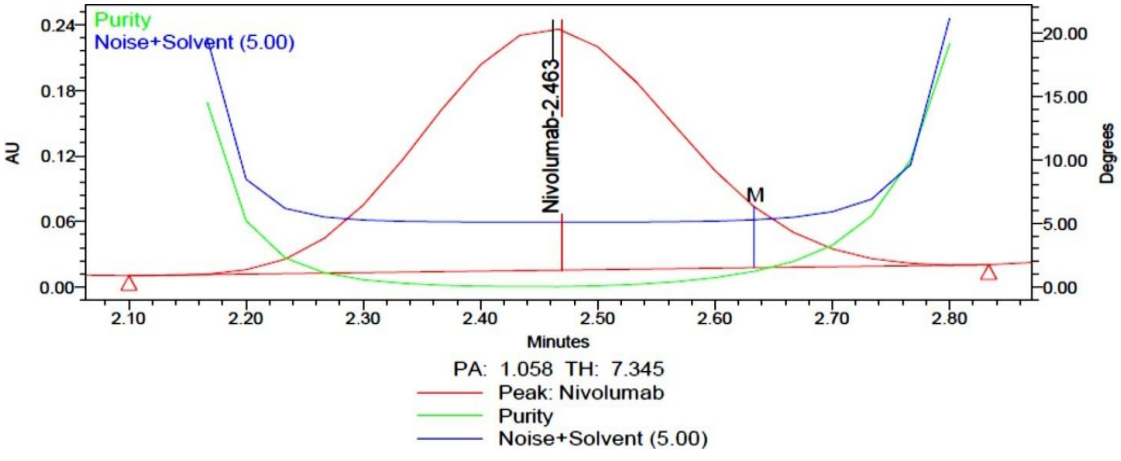

Fig.26: Purity Plot of Nivolumab

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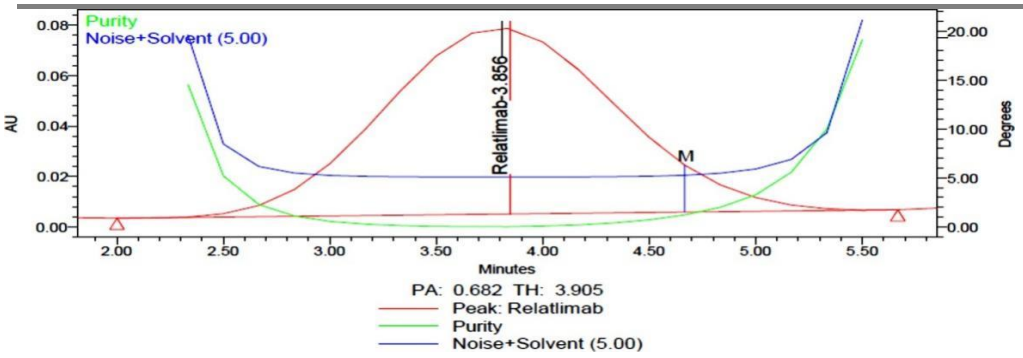

Fig.27: Purity Plot of Relatlimab

Percentage assay

Percentage assay of the Nivolumab and Relatlimab in injection that were found as 100% ± 0.2 indicates that the analyzed injection have percentage purity within the acceptance limits as per ICH guidelines. Results were shown in Table 8.

Table. No:8 Assay of Nivolumab and Relatlimab

Brand	Drug	Avg sample area (n=2)	Std. Conc. (µg/ml)	Sample Conc. (µg/ml)	Label amount (mg)	Std purity	Amount found (µg/ml)	% Assay
Opudulag	Nivolumab	2645101	120	120	240	99.9	119.5	99.9
	Relatlimab	891390	40	40	80	99.9	39.8	99.8

DISCUSSION

The stability indicating RP-HPLC assay method plays a significant role in determination of intrinsic stability, both qualitative and quantitative estimation of drug product and drug substance. Till date, only very few analytical methods have been developed for Nivolumab and Relatlimab individual and in combination with other anti-cancer drugs. But, no RP-HPLC method has been existed for the stability indicating simultaneous estimation of Nivolumab and Relatlimab. Hence, attempts were made to develop an effective stability indicating RP-HPLC method. The RT in the reported method was 2.456 min for Nivolumab and 3.884 min for Relatlimab, represents the method with good and effective retention time, and can be treated as economical as it reduces solvent consumption and analyte run time. Hence, rapid analysis of more number samples can be done. The calculated and statistical results of the validation parameters were not out of the acceptance limits stated by ICH.

CONCLUSION

A simple, accurate, sensitive, and specific RP HPLC with PDA detector and isocratic elution method was successfully developed for the simultaneous estimation of Nivolumab and Relatlimab in bulk and its formulation. Forced degradation studies were done by applying several stress conditions to assess the stability of the method. The proposed method was successfully separate both of the drugs and its degradation products with good resolution and quantifies the active contents at minute concentration levels. The developed method has specific, sensitive, and stability-indicating power. Hence, the proposed method can be adapted to regular analysis in pharmaceutical industry

Abbreviations

TFA: Trifluoroacetic acid; RT: Retention time; LOD: Limit of detection; LOQ: Limit of quantification; SD: Standard deviation; RSD: Relative standard deviation; PDA: Photodiode array; NMB: Nivolumab; RMB: Relatlimab;

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Authors contributions

All contributed equally in design of the work, acquisition and interpretation of data, and manuscript preparation. All authors have read and approved the manuscript.

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Availability of data and materials

All data and materials should be available upon request.

Ethics approval and consent to participate

No animals and human subjects used in this study.

Consent for publication

Not applicable

Competing interests

The authors declare that there is no conflict of interest

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