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SIMULTANEOUS ESTIMATION OF THE NIVOLUMAB AND RELATLIMAB IN BULK AND PHARMACEUTICAL DOSAGE FORM BY USING RP-HPLC.

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ABSTRACT: A simple, Accurate, precise method was developed for the simultaneous estimation of the Nivolumab and Relatlimab in Tablet dosage form. Chromatogram was run through Standard Agilent C18 (150mmX 4.8mm, X5µ). Mobile phase containing 0.01N Ammonium acetate Buffer and Acetonitrile in the ratio of 60:40 was pumped through column at a flow rate of 1.0 ml/min. Temperature was maintained at 30°C. Optimized wavelength for Nivolumab and Relatlimab was 221nm. Retention time of Nivolumab and Relatlimab were found to be 2.252 min and 2.751 min. %Recover was Obtained as 100.01% and 99.87% for Nivolumab and Relatlimab. LOD, LOQ values were obtained from regression equations of Nivolumab and Relatlimab were 0.02ppm, 0.07 ppm and 0.03ppm, 0.09ppm respectively. Regression equation of Nivolumab is y = 43453x + 2222.4, and of Relatlimab is y = 29211x + 2391.5. Retention times and run time was decreased so the developed method was economical which can be adopted in regular basis test.

Key Words: Nivolumab, Relatlimab, RP-HPLC.

INTRODUCTION

Melanoma is a type of skin cancer that starts with the cells called melanocytes which was mainly responsible for the pigment control in skin. People diagnosed with advanced melanoma have a new treatment option like immunotherapy with checkpoint inhibitors. In a large clinical trials people with advanced melanoma treated with combination of two immunotherapy drugs.

A double-drug combination of Nivolumab and Relatlimab is one of the fixed-dose combinations which are very effective and widely used due to their immunotherapy property.

Nivolumab is indicated to treat unresectable or metastatic melanoma, melanoma as adjuvant treatment, re-selectable or metastatic non-small cell lung cancer. It mainly worked by the ligands PD-L1 and PD-L2 bind to the PD-1 receptor on T-cells, inhibiting the action of these cells. Tumor cells express PD-L1 and PD-L2.[6] Nivolumab binds to PD-1, preventing PD-L1 and PD-L2 from inhibiting the action of T-cells, restoring a patient's tumor-specific T-cell response.

Relatlimab is the first developed anti-LAG-3 antibody which enters the clinical trials in 2013, and has used in the treatment of a variety of cancers, including leukemia and melanoma. As immune checkpoint inhibitors it has very limited efficacy when it used alone, drugs like Relatlimab have been trialed in combination with other checkpoint inhibitors like nivolumab. It worked by Lymphocyte-activation gene 3 (LAG-3) - formally known as CD223 - is a type I transmembrane protein which belongs to the immunoglobulin superfamily. [1,6] Its expression on activated T-cells was mainly induced to antigen stimulation,[3] and they serve a number of functions including the inhibition of Th1 cell proliferation and the reduction of cytokine production - such as IL-2, IFNγ, and TNF - in these activated T-cells. Relatlimab is a human IgG4 monoclonal antibody that binds

LAG-3 and inhibits it signaling pathway, the antagonism of which promotes T-cell proliferation, cytokine secretion, and, subsequently, restored tumor immunosurveillance. Used in combination with nivolumab, a PD-1 receptor blocker, Relatlimab has been shown to potentiate the anti-tumor effects of PD-1 blockade.

The quality and safety measurements of a drug is generally assured by monitoring and controlling the assay and impurities effectively. The assay determines the potency of the drug product and impurities which covers the safety aspect of the drug. The assay of pharmaceutical products plays a major role in the efficacy of the drug in patients.

A wide variety of challenges is encountered while developing the methods for different drugs depending on their nature and properties for achieving the selectivity, speed, cost, simplicity, sensitivity, reproducibility, and accuracy. Which allows researchers to come out with solutions to address the challenges. The physicochemical methods mainly optical (refractometry, polarimetry, emission, and fluorescence methods of analysis), photometry (photocolorimetry and spectrophotometry covering UV-Visible, IR Spectroscopy, and nephelo-turbidimetry), and chromatographic (column, paper, thin layer, gas-liquid, and high-performance liquid chromatography) methods. Methods such as nuclear magnetic resonance (NMR) and para-magnetic resonance (PMR) and the hyphened methods (GC-MS) are becoming more and more popular. **DRUG PROFILE**

Relatlimab

CAS number: 946414-94-4

Chemical Formula: C6472H9940O2026N1704S38

Purity: ≥98%

Molecular Weight:143597.3811 D

Technical Information

Appearance: Powder

Physical State: Liquid

Storage at: -20° C

Melting Point: 135.5° C

pKa Values: 6.5

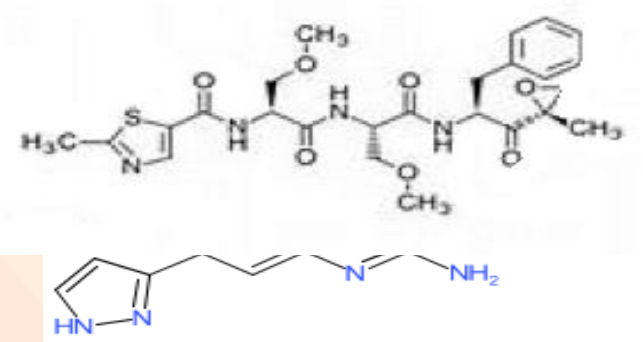

Fig1: Structure of Relatlimab

Nivolumab:

CAS Number: 946414-94-4

Molecular Weight: 143597.3811 Da

Molecular Formula: $C_{6362}H_{9862}N_{1712}O_{1995}S_{42}$

Appearance: White powder

Physical State: Liquid

PKA: 5.8

Solubility: Soluble in ethanol methanol

MATERIALS AND METHODS

Materials:

Combination of Nivolumab and Relatlimab Injection (**Opdualag**) dosage forms, distilled water, acetonitrile, phosphate buffer, ammonium acetate buffer, glacial acetic acid, methanol, potassium dihydrogen phosphate buffer, tetrahydrofuran, triethylamine, ortho-phosphoric acid etc.

Methods

Preparation of buffer:

Buffer: 0.01N Ammonium acetate

Accurately weighed 0.77gm of Ammonium acetate in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water.

Buffer: (0.1% OPA)
Accurately 1ml of OPA in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water.
Preparation of Standard stock solutions: Accurately weighed 24mg of Nivolumab, 8mg of Relatlimab was transferred and prepare Standard stock solution of 480µg/ml of and 160µg/ml. From the solution working standard was prepared 18µg/ml of Nivolumab and 16µg/ml of Relatlimab.

Sample Preparation:
Accurately take 20ml of liquid and the volume made up 100ml with diluent and filtered that gives 2400µg/ml of Nivolumab and 800µg/ml Relatlimab. From the solution working standard was prepared 18µg/ml of Nivolumab and 16µg/ml of Relatlimab.

Validation

System Suitability: The standard solution of 18µg/ml of Nivolumab and 16µg/ml of Relatlimab were prepared, and solutions were injected six times to determine parameters like peak tailing, resolution, and USP plate count.

The % RSD for the area of six standard injections results should not be more than 2%.

Specificity: Checking for interference in a method that has been optimized. At the retention times of these drugs in this approach, we should not detect interference peaks in blank and placebo samples. So, it was stated that this approach was specific.

Linearity: The linearity of the method was estimated by preparing calibration samples which were prepared by Spiking appropriate amount of Nivolumab and Relatlimab in acetate buffer and acetonitrile to give 12-72 and 4-24 ppm.

Limit of detection (LOD) and Limit of quantification (LOQ): The Limit of detection (LOD) and limit of quantification (LOQ) was determined according to the ICH guidelines for the validation of analytical procedure. The formulae used were:

$$LOD = 3.3\sigma/S$$

$$LOQ = 10\sigma/S$$

Where, σ = standard deviation of the response (intercept)

S = slope of the calibration curve

Accuracy: Accuracy of the method was determined at three different levels (50%, 100%, and 150%) mean and %RSD values were calculated. The % RSD for the area of six standard injections results should not be more than 2%.

Robustness: Small deliberate adjustments were made to the procedure, such as flow rate, mobile phase ratio, and temperature, but there was no noticeable variance in the outcome and it remained within the ICH guideline range. Robustness conditions like Flow minus (0.9ml/min), Flow plus (1.1ml/min), mobile phase minus, mobile phase plus, temperature minus (25°C) and temperature plus (35°C) was maintained and samples were injected in duplicate manner. The % RSD for should not be more than 2%.

Degradation studies:

Oxidation:

To 1 ml of stock solution of Nivolumab and Relatlimab, 1 ml of 20% hydrogen peroxide (H2O2) was added separately. The solutions were kept for 30 min at 60^0c. For HPLC study, the resultant solution was diluted to obtain 48µg/ml &16µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation Studies:

To 1 ml of stock ssolution Nivolumab and Relatlimab, 1 ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60^0c. The resultant solution was diluted to obtain 48µg/ml & 16µg/ml solution and 10 µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies:

To 1 ml of stock solution Nivolumab and Relatlimab, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 60^0c. The resultant solution was diluted to obtain 48µg/ml & 16µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation Studies:

The standard drug solution was kept in oven at 105°C for 6 h to study dry heat degradation. For HPLC study, working solution of 10µl were injected into the system and record the chromatograms to see the stability of the sample**.**

Photo Stability studies:

The photochemical stability of the drug was studied by exposing the 200µg/ml& &100µg/ml solution to UV Light for 7days or 200Watt hours/m^2 in photo stability chamber For the study, the solution was diluted to 20µg/ml &10µg/ml and 10 µl was injected into the HPLC and record the chromatograms to check the stability of sample.

Neutral Degradation Studies:

Stress testing was studied by refluxing the drug in water for 6hrs at a temperature of 60º. Then the resultant solution was diluted to working standard and 10 µl was injected into the HPLC and record the chromatograms to check the stability of the sample**.**

RESULTS AND DISCUSSION

Method development: method was development was done by changing various mobile phase ratio, buffers etc.

Optimized Chromatogram: The chromatography elution was carried out in the isocratic mode where the mobile phase was selected as 0.01N Ammonium acetate: Acetonitrile (60:40) ratio. The flow rate of the mobile phase was selected as 1 ml/min with a run time of 5 min at 30°C temperature. Injection volume is 10µL.The eluent was passed through Agilent (4.6 x 250mm, 5µm) and monitoring by using UV detector at 221nm.

System suitability: The system found to be suitable and within range and it satisfy the ICH guidelines. According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits.

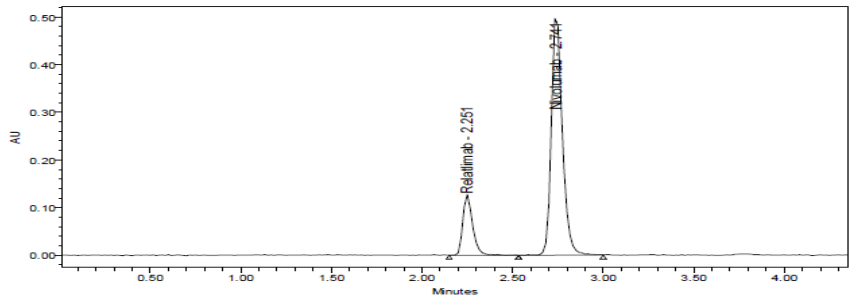

Fig3: Typical chromatogram of Nivolumab and Relatlimab.

Table1: System suitability studies of Nivolumab and Relatlimab method

Sl. no	Relatlimab			Nivolumab			
Inj	RT (min)	USP Plate Count	Tailing	RT (min)	USP Plate Count	Tailing	Resolution
1	2.242	7339	1.25	2.736	9383	1.19	4.5
2	2.248	7816	1.22	2.739	9681	1.17	4.6
3	2.253	7382	1.26	2.747	9831	1.17	4.6
4	2.253	7340	1.26	2.749	9470	1.17	4.7
5	2.256	7898	1.24	2.752	9581	1.18	4.5
6	2.257	7873	1.23	2.753	9536	1.19	4.5

Linearity: Six Linear concentrations of Nivolumab (12ppm-72ppm) and Relatlimab (4ppm to 24ppm) are prepared and injected. Regression equation of the the Nivolumab and Relatlimab are found to be, y = 43453x + 2222.4 and y = 29211x + 2391.5 and the regression co-efficient was 0.999.

Table2: Calibration data of Nivolumab and Relatlimab method.

Sl.no	Concentration Nivolumab (µg/ml)	Response	Concentration Relatlimab (µg/ml)	Response
1	0	0	0	0
2	12	528786	4	114859
3	24	1048786	8	235689
4	36	1548518	12	368416
5	48	2098500	16	466994
6	60	2615449	20	583451
7	72	3125762	24	701040

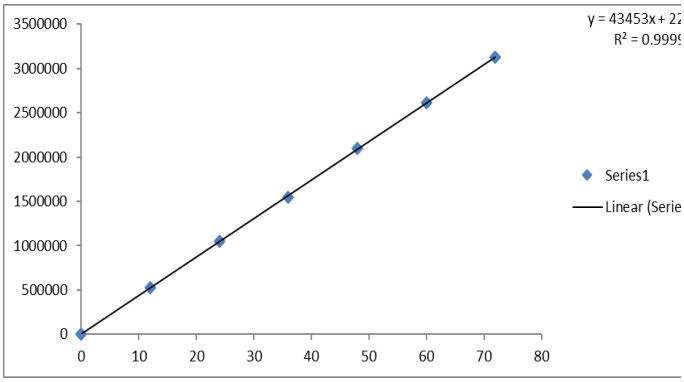

Fig4: Calibration curve of Nivolumab

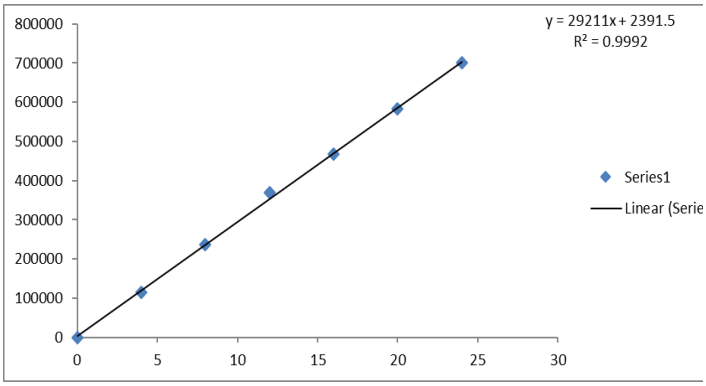

Fig5: Calibration curve of Relatlimab

Precision:

Intraday precision (Repeatability): Intraday Precision was performed and % RSD for Nivolumab and Relatlimab were found to be 0.4% and 0.4% respectively.

Table3: Repeatability results for Nivolumab and Relatlimab.

Sr. No.	Nivolumab	Relatlimab
1	2084785	469038
2	2073534	463712
3	2067139	464826
4	2089451	468697
5	2076875	460974
6	2055238	465670
Mean	2074504	465486
Std. Dev.	12337.7	3062.7
%RSD	0.6	0.7

Inter day precision: Inter day precision was performed with 24 hrs time lag and the %RSD Obtained for Nivolumab and Relatlimab were 1.2% and 0.3%.

Table4: Inter day precision results for Nivolumab and Relatlimab.

Sr. No.	Nivolumab	Relatlimab
1	2050794	462388
2	2070909	462334
3	2078780	469338
4	2059630	463125
5	2062594	460866
6	2088825	469058
Mean	2068125	464518
Std. Dev.	15371.6	3699.4
%RSD	0.7	0.8

Accuracy: Accuracy was performed by checking the sample concentrations at three different range 50%, 100%, 150%. The sample were injected in a triplicate manner and amount Recovered and % Recovery was seen.

Table5: Accuracy results of Nivolumab

% Level	Amount Spiked (µg/mL)	Amount recovered	%Recovery	Mean %Recovery
50%	24	24.11	100.47	100.01%
	24	24.13	100.54	
	24	23.95	99.78	
100%	48	47.37	98.69	
	48	48.47	100.98	
	48	47.75	99.48	
150%	72	72.33	100.46	
	72	71.47	99.27	
	72	72.32	100.45	

Table 6: Accuracy results of Relatlimab

% Level	Amount Spiked (µg/mL)	Amount recovered	%Recovery	Mean %Recovery
50%	8	8.05	100.57	99.87%
	8	7.99	99.91	
	8	8.05	100.57	
100%	16	15.86	99.15	
	16	15.90	99.37	
	16	16.00	100.03	
150%	24	24.16	100.65	
	24	23.81	99.23	
	24	23.85	99.38	

LOD: LOD for Nivolumab was found to be 0.02 and Relatlimab was 0.03 respectively.

LOQ: LOQ for Nivolumab and Relatlimab were found to be 0.07 and 0.09 respectively.

Robustness: Small deliberate changes in method like Flow rate, mobile phase ratio, and temperature are made but there was no recognized change in the result and are within range as per ICH Guide lines.

Table7: Robustness data of Nivolumab and Relatlimab method.

Sl. No	Robustness condition	Nivolumab %RSD	Relatlimab %RSD
1	Flow minus	0.6	0.8
2	Flow Plus	0.7	1
3	Mobile phase minus	0.6	0.6
4	Mobile phase Plus	0.3	0.8
5	Temperature minus	1	0.4
6	Temperature Plus	0.6	0.3

Assay: To perform the assay standard preparations and Sample Preparations are prepared. Both of them sample and standards are injected in six homogeneous form. Drug in the formulation was estimated by comparing with the results with standard which was used as reference. The Average of the Assay was taken and it was found to be 100.08% and 99.93% for Nivolumab and Relatlimab respectively.

Table8: Assay of Tablet

S. No.	Nivolumab %Assay	Relatlimab %Assay
1	100.58	100.69
2	100.03	99.54
3	99.72	99.78
4	100.80	100.61
5	100.19	98.96
6	99.15	99.96
AVG	100.08	99.93
STDEV	0.595	0.66
%RSD	0.6	0.7

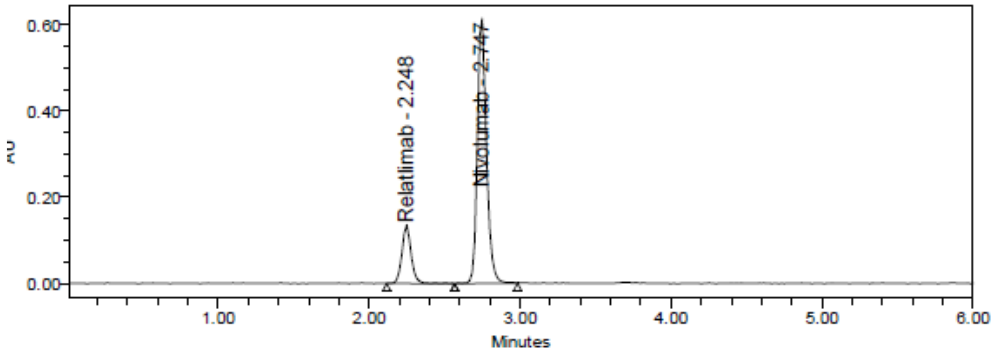

Fig6: Assay Chromatogram of Sample

Table9: Degradation data

Type of degradation	Nivolumab			Relatlimab		
	Area	%recovered	% degraded	Area	%recovered	% degraded
Acid	1915865	92.43	7.57	436906	92.79	7.21
Base	1910344	92.16	7.84	437439	92.91	7.09
Peroxide	1925197	92.88	7.12	437706	92.96	7.04
Thermal	2020530	97.48	2.52	457697	97.21	2.79
UV	2026063	97.74	2.26	458174	97.31	2.69
Water	2054419	99.11	0.89	466208	99.02	0.98

SUMMARY

Table 8: Summary Table

Parameters	Nivolumab	Relatlimab
Calibration range (mcg / ml)	12-72 ppm	4-24ppm
Optimized wavelength	221nm	221nm
Retention time	2.252min	2.751min
Regression equation (Y)	y = 43453x + 2222.4	y = 96186x + 3504
Correlation coefficient(r^2)	0.999	0.999
Precision (% RSD*)	0.9	0.6
% Recovery	100.01	99.87
Limit of Detection (µg/ ml)	0.02	0.03
Limit of Quantitation (µg / ml)	0.07	0.09

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