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SIMULTANEOUS QUANTIFICATION OF HUMAN MONOCLONAL ANTIBODIES: RELATLIMAB and NIVOLUMAB BY RP-HPLC METHOD

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ABSTRACT

The aim of the current investigation is the simultaneous quantitation of Nivolumab and Relatlimab in a pure form as well as in formulation. The chromatographic separation was achieved on Agilent (4.6 mm × 150 mm; 5µm), with a combination of Acetonitrile and ortho-phosphoric acid (0.1%) in a proportion of 45:55 v/v. Flow rate is One mL/minute, the detection wavelength at 221 nm. Analysis of linearity within a sample strength in range of 12 - 72 ppm of Relatlimab and 4-24 ppm of Nivolumab with correlation coefficients of 0.9998 and 0.9999, respectively. Relatlimab and Nivolumab retention times - 2.470 min. and 3.423 min., correspondingly. Relatlimab and Nivolumab LoD's, LoQ's were 0.08 ppm, 0.26 ppm and 0.03 ppm, 0.08 ppm, respectively. The method was validated for selectivity, specificity, accuracy, linearity, range, precision, quantification limit and limit of detection, and robustness. Stability indicating studies were performed by Acid, Alkali, peroxide, thermal, UV light and water.

Keywords: Simultaneous, Relatlimab, Nivolumab, Validation, Accuracy, Precision.

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INTRODUCTION

Several studies have demonstrated that, comparison with higher dose monotherapy, the components of combination treatment may be provided at lower doses, have a decreased risk of adverse side effects. Combination treatment, therefore, may increase patient adherence and popularity.[1-5] A drug called Opdualag is a fixed dose combination of human monoclonal antibodies Relatlimab (RLT) and Nivulomab (NVM), a group of medicines used to treat tumors as well as other malignancies.[6] OpdualagTM is available in a 20 mL single-dose vial consisting of 240 mg - NVM and 80 mg - RLT for intravenous injection. The solution is clear to opalescent, colourless to slightly yellow. It is absent from all official pharmacopoeial monographs, though. Consequently, developing a novel chromatographic technique for quantification of NVM, RLT in marketed dosage forms is essential. One common human monoclonal antibody (mAb) used to treat melanoma[7,8] is OPDUALAG, a fixed combination of Nivolumab and Relatlimab. This group includes lung cancer, melanoma, kidney cell carcinoma, Hodgkin's disease, cancer of the neck, head, liver, and colon. It was found at Medarex[9], developed along with Ono Pharmaceutical, and then Bristol-Myers Squibb[10] and Ono brought it to the market. It is administered slowly into a vein, and adverse effects have been seen to include fatigue, rash, liver issues, muscular aches, and cough.[11] Melanoma (a kind of cancer of the skin) that has spread or cannot be surgically excised is treated with it as a first-line option. Patients from the age of 12 who have cancer cells that

generate just 1% of a protein called PD-L1[12]. The target protein, known as the programmed death 1 receptor (PD-1), is bound by Nivolumab. A lymphocyte activation gene 3 (LAG 3), known as the target protein, is where Relatlimab binds. Thymocytes, a subclass of white blood cells (WBC) that are a part of the defense system, the body's natural défense, can have their activity turned off by those proteins. Nivolumab and Relatlimab disrupt the activities of LAG 3 and PD 1 and stop them from turning off your T cells by binding to them. By doing so, their action against the melanoma cancer cells is increased by[13]. Relatlimab molecular weight is 349.17 g/mol and is designated as "*N-((4-amino-7-(1H-pyrazole3-yl)-1H-imidazo[4,5-c] quinoli-2-yl) methy)-N-ethyl acetamide*"[14] and Nivolumab molecular weight is 532.61 g/mol and is chemically designated as "*N-(3-methoxy-1-((3-methoxy-1 - ((1 -(2 – methyloxiran – 2 - yl) – 1 - oxo-3-phenylprpoan-2-yl) amino) -1-oxopropan -2-yl) amino) - 1-oxopropan - 2 - yl)-methylthiazole-5-carboxamide*".[15] Literature review reveals that the availability of published data on these drugs in HPLC[16,17,18], UPLC-MS/MS[19], LC-MS[20], UPLC[21], Bioanalytical LC method with other combination drugs and alone. There may not be other published work on concurrent determination of Relatlimab and Nivolumab in pure form and commercial formulation. As a result, an effort has been made to design an easy-to-use, accurate, and affordable RP-HPLC technique to assess both marketed formulation and bulk drug.

EXPERIMENTAL

Materials and Methods

The study was carried out using a quarternary pump, PDA detector (photodiode array detector), 2996 on a RP-HPLC system - Water Alliance e-2695. Data collection made by the chromatography software Empower 2.0. HPLC-grade materials like water, phosphoric acid (OPA), and acetonitrile were procured from Merck India Ltd., Bombay. The RLT and NVM reference standards' active pharmaceutical ingredients (APIs) were developed by Mumbai, India's Glenmark Pharmaceuticals Private Ltd.

Chromatographic Conditions

Isocratic elution was used for the chromatographic analysis, and the mobile phase was buffer: acetonitrile (0.1% OPA) (45:55 v/v) ratio. Elution components were found at 221 nm wavelength, and the flow rate of mobile phase was set at one millilitre per minute and volume of injection was 10 µL and a total run time was 6 min.

Preparation of Standard Solution

Relatlimab and Nivolumab were weighed, placed in a 50 mL graduated flask. After that, diluent 10 mL was added, allowed to sonicate for 10 min., then make up the volume with diluent. (480 ppm of Nivolumab and 160 ppm of Relatlimab). 1mL of the sample solution was placed in a 10 mL graduated flask, and then the diluent was added up to the mark. (16 ppm of Relatlimab and 48 ppm of Nivolumab).

Preparation of Sample Solution

The combined sample was precisely weighed before being placed into a 100 mL volumetric flask. The mixture was sonicated for 25 minutes after being diluted with 50 mL of diluents. Finally, make up the volume with diluent, purified using HPLC filters (containing 2400 ppm of Nivolumab and 800 ppm of Relatlimab). A 10 mL volumetric flask was used to dilute 0.2 mL of the sample stock solution of Relatlimab (16 ppm) and Nivolumab (48 ppm).

Method Validation

This approach validates system suitability criteria for the selected Relatlimab and Nivolumab drugs, including accuracy, linearity, precision, LOQ, LOD, robustness and stress testing studies.

System Suitability

The prepared standard solutions were introduced into the chromatographic system and determine the system suitability variables lie within the limits or not.

Linearity

The procedure's capacity to yield findings that, within acceptable limits, are directly or indirectly related to sample concentration.

Precision
The level of close agreement between test results obtained using different samples of a same sample. It is used to estimate how repeatable or reproducible a procedure is (its capacity to work in identical or unlike circumstances).

Accuracy
To obtain the results, a method that was close to the real value was used.

LOQ and LOD
For each analyte, the quantification limit, detection limit was determined using the signal/ noise ratio.

Robustness
By making small adjustments to the experimental variables, such as organic content and flow rate, the robustness approach was evaluated. Robustness uses the same equipment at various chromatographic conditions.

Forced Degradation Studies
Stress testing studies are carried out to ascertain the drug analyte's stability under different conditions, namely acidic degradation, alkaline, thermal, oxidation and UV light. Acidic degradation by 2N hydrochloric acid, alkali degradation by 2N sodium hydroxide and thermal degradation are accomplished by heat. A 20% hydrogen peroxide solution is used for the oxidation degradation examination. Photolytic degradation is accomplished by UV light. The medication was refluxed in water to study stress testing in neutral conditions for 6 hours at 60^0 C.

RESULTS AND DISCUSSION

Table-1: Optimized Chromatographic Conditions

Parameter	Value
Column	Agilent (4.6 mm x 15 cm, 5µm)
Mobile Phase	Acetonitrile (ACN): Buffer (45:55 v/v)
Injection Volume	10 µL
Rate of Flow	1 mL/ min.
Column Temperature	30 min.
Wavelength	221 nm
Time duration	6 minutes
Retention time of Relatlimab	2.470 min.
Retention time of Nivolumab	3.423 min.

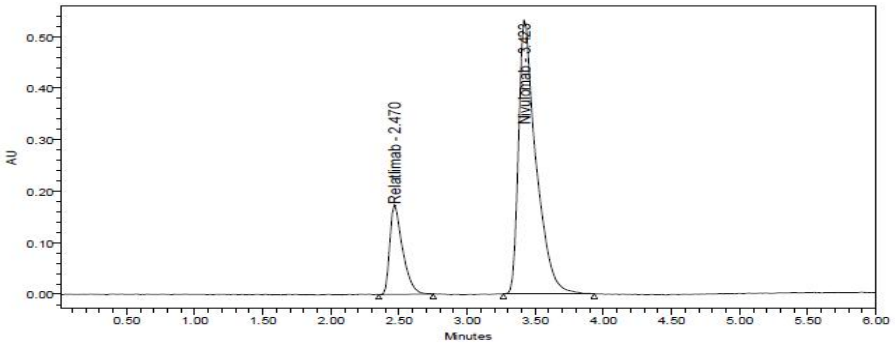

Fig.-1: Optimized Chromatogram of Relatlimab and Nivolumab

System Suitability
Each System suitability variable lies within acceptable ranges.

Linearity
The linearity technique was assessed by making a standard solution comprising 48 ppm of Relatlimab and 16 ppm of Nivolumab. The outcomes are displayed in Table-2, Fig.-2 and Fig.-3.

Table-2: Linearity Result

S. No.	Relatlimab		Nivolumab	
	Concentration (ppm)	Peak Response	Concentration (ppm)	Peak Response
1	12	268289	4	883400
2	24	536441	8	1766822
3	36	805722	12	2652230
4	48	1071776	16	3537875
5	60	1345406	20	4412212
6	72	1583050	24	5227901

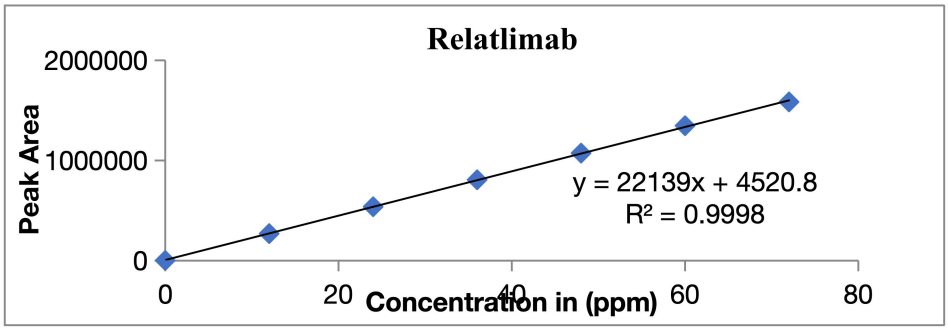

Fig.-2: Calibration Curve of Relatlimab

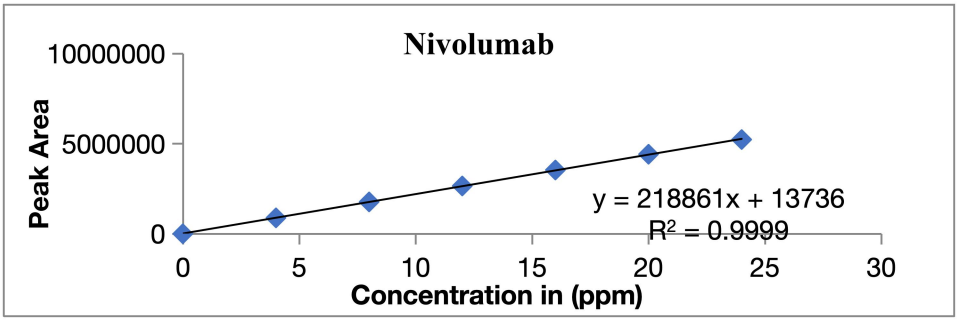

Fig.-3: Calibration Curve of Nivolumab

LOD and LOQ

Relatlimab and Nivolumab, LOD values - 0.08 and 0.03 ppm, correspondingly. Relatlimab and Nivolumab, LOQ values - 0.26 and 0.08 ppm, correspondingly.

Precision

The method's accuracy was examined by the observation of 06 different samples from the homogenous batch. The current approach was proven to be accurate since the assay percentage values were close to 100% and the RSD was less than 2%. The outcomes were tabulated in Table-3.

Table-3: Intraday and Interday Precision of Relatlimab and Nivolumab

S. No.	Relatlimab	Nivolumab	Relatlimab	Nivolumab
1	1086091	3551944	1077010	3511656
2	1076950	3563605	1059338	3579749
3	1078428	3555098	1060920	3573554
4	1082536	3541285	1073477	3524308
5	1075972	3502659	1075595	3541366
6	1071190	3527653	1052134	3555269
Mean	1078528	3540374	1066412	3547650
SD	5218.1	22250.5	10301.7	26978.9
% RSD	0.5	0.6	1.0	0.8

Accuracy

Accuracy recovery checks were determined in three distinct concentration levels (150%, 100%, and 50%). Table-4 and Table -5 shows the accuracy results.

Table-4: Relatlimab Accuracy Results

Accuracy - Relatlimab				
Level in %	API Added Amount (mg)	Recovered Amount (mg)	Recovery %	Average % Recovery
50	24	24.0	100.2	100.14
	24	23.8	99.0	
	24	23.9	99.6	
100	48	48.4	100.8	
	48	48.3	100.7	
	48	47.8	99.6	
150	72	72.0	100.0	
	72	72.5	100.7	
	72	72.5	100.7	

Table-5: Nivolumab Accuracy Results

Accuracy - Nivolumab				
Level in %	API Added Amount (mg)	Recovered Amount (mg)	Recovery %	Average % Recovery
50	8	8.04	100.45	100.48
	8	8.03	100.34	
	8	7.97	99.63	
100	16	15.99	99.93	
	16	16.11	100.70	
	16	16.14	100.88	
150	24	24.23	100.95	
	24	24.15	100.61	
	24	24.19	100.80	

Robustness

The results of robustness are displayed in Table-6.

Table-6: Results of Robustness

Parameter	Relatlimab	Nivolumab
Flow (+)	0.8	0.4
Flow (-)	0.9	0.5
Mobile phase (+)	0.8	0.7
Mobile phase (-)	1.0	0.6
Temperature (+)	0.9	0.5
Temperature (-)	0.5	0.3

Table-7: Results of Degradation Studies

Degradation	% Deg. of Relatlimab	% Deg. of Nivolumab
Acid	4.92	4.51
Alkali	4.32	4.82
Peroxide	6.59	5.30
Thermal	2.87	2.91
Photo	1.83	1.21
Hydrolysis	0.44	0.53

CONCLUSION

For the simultaneous quantification of Relatlimab and Nivolumab in pharmaceutical dose form as well as pure form, the suggested RP-HPLC technique was created and validated. The suggested method's properties, such as linearity, precision, accuracy, robustness, limit of quantification and limit of detection, were tested as per the guidelines of ICH Q2(R1).

The suggested, verified stability-indicating method's efficiency was demonstrated by the forced degradation studies. The newly developed method, which may be used in routine internal control testing in the pharmaceutical companies, was accurate, sensitive, and economic.

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CONFLICT OF INTERESTS

The authors stated that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

The present work is related to *SDG-3: Good Health and well-being*. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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