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Research Article

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF NIVOLUMAB AND RELATLIMAB IN PHARMACEUTICALS DOSAGE FORM BY HPLC

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ABSTRACT
The pharmaceutical dosage forms of the immunotherapy treatment medicine Nivolumab and Relatlimab were identified using high-performance liquid chromatography (HPLC). A 150 x 4.6 mm Agilent C 18 column with a particle size of 5µm was utilized. Sixty percent ammonium acetate buffer and forty percent acetonitrile make up the mobile phase. A wavelength of 221 nm was identified. Nivolumab had a retention of 2.245 minutes and Relatlimab of 2.736 minutes. The relative standard deviations of the two medications were 0.5% and 0.3%, respectively; the regression lines for the two drugs were y = 76535x + 17268 and y = 78171x + 5761.7, and the Assay results for each drug were 99.64% and 99.91%, respectively. All the other metrics were verified and monitored within the specified ranges.
Key words: Nivolumab, Relatlimab, Rp Hplc, Validation.

INTRODUCTION
Nivolumab and relatlimab are monoclonal antibodies used in cancer immunotherapy, specifically targeting immune checkpoint pathways to enhance anti-tumor responses. Nivolumab is a programmed death-1 (PD-1) inhibitor that prevents the interaction between PD-1 and its ligands PD-L1 and PD-L2. This interaction blockade reactivates T cells, allowing them to attack cancer cells more effectively [1]. Relatlimab is an inhibitor of lymphocyte activation gene-3 (LAG-3), a negative regulator of T cell activation. By targeting LAG-3, relatlimab promotes T cell proliferation and enhances immune activity against tumor cells [2]

The combination of nivolumab and relatlimab, marketed as Opdualag, provides a dual checkpoint blockade approach. This synergy enhances the immune system's capacity to eliminate cancer cells more effectively compared to single-agent therapies [3]. In March 2022, the U.S. Food and Drug Administration (FDA) approved this combination for treating unresectable or metastatic melanoma in patients aged 12 years and older, based on the pivotal RELATIVITY-047 trial [4]. The trial demonstrated a significant improvement in progression-free survival with the combination therapy compared to nivolumab alone [3]

This therapeutic strategy is significant for its potential to overcome resistance mechanisms seen in monotherapy and represents a novel milestone in the field of immuno-oncology. Current research is investigating the efficacy of nivolumab and relatlimab in other cancers to broaden their clinical applications [5]

Nivolumab: Nivolumab is a PD-1 blocking antibody used to treat melanoma, non small-cell lung cancer, renal cell cancer, head and neck cancer, and Hodgkin lymphoma. [6]

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Relatlimab: Relatlimab is a monoclonal antibody targeted against LAG-3 which is used in combination with nivolumab for the treatment of unresectable or metastatic melanoma.[7]

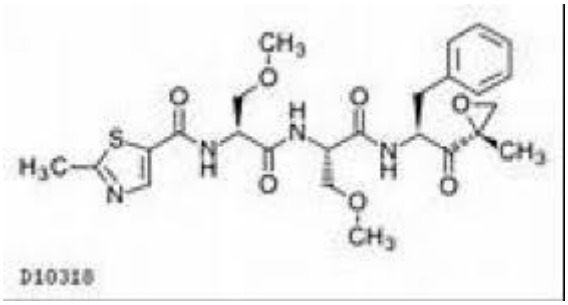

Figure 1: Structure of Nivolumab

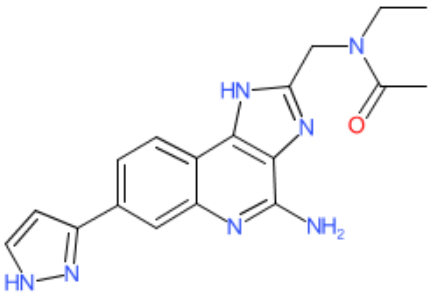

Figure 2: Structure of Relatlimab

Extensive literature research has unearthed a multitude of recorded analytical procedures, including the discovery of more economically efficient ways. Hence, a reliable and cost-effective approach is suggested for assessing the stability of Nivolumab, Relatlimab, and their medicinal dose form using RP-HPLC.[8-12] must be validated and developed as per ICH guidelines

MATERIALS AND METHODS

Spectrum pharma Research Solution provide with Nivolumab and Relatlimab pure drugs (API) gift samples and Combination Nivolumab and Relatlimab received from local market. The chemicals and buffers utilized in this estimation were obtained from Rankem, an Indian supplier.

Instrumentation:

The development and method validation were conducted using a WATERS HPLC, specifically the model 2695 SYSTEM, equipped with a Photo diode array detector. The system also included an automated sample injector and the Empower 2 software.

Objective:

The primary objective of this study is to provide a highly exact, accurate, sensitive, specific, consistent, and efficient analytical method for the simultaneous quantification of Nivolumab and Relatlimab in their pure form and throughout tablet formation.

Preparation:

Standard solution: 24 milligrams of Nivolumab and 8 mg of Relatlimab were weighed and thereafter added to 50 ml vf. Subsequently, the volumetric flask is filled to the neck with the diluent, which was solubilized using sonication for 10 minutes. After the medication was dissolved, the diluent was included into the volume fraction until it reached the specified concentration (Relatlimab - 160µg/ml, Nivolumab - 480µg/ml). Subsequently, 1 ml of stock solution was introduced into a 10 ml vial containing diluent, and the mixture was brought to the appropriate volume. (Nivolumab - 48 µg/ml, Relatlimab - 16 µg/ml).

Sample Solution: Precisely measure 20 mL of liquid solution and put it into a 100 mL volumetric flask. Add 50 mL of diluent and sonicate for 25 minutes. Subsequently, adjust the volume with diluent and filter the solution. (Relatlimab- 800µg/ml, Nivolumab - 2400µg/ml), 0.2 ml of the stock solution was spiked and added to a 10-milliliter volumetric flask, then diluted to the mark with a diluent. (Relatlimab - 16 µg/ml, Nivolumab - 18 µg/ml).

System suitability parameters:

The system suitability parameters were determined by preparing standard solutions of Nivolumab (48ppm) and Relatlimab (16ppm) and the solutions were injected six times and the parameters like peak tailing, resolution and USP plate count were determined.

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

Specificity: Checking of the interference in the optimized method. We should not find interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Table 1: Chromatographic Conditions:

Mobile phase	0.01NAmmonium acetate: Acetonitrile (60:40)
Column used	Agilent C18 Column, 5 µm, 4.6 x 250 mm
Stream rate	1 ml/min
λ max	221
C Temp	30°C
Inj	10 µL
Range	10 min

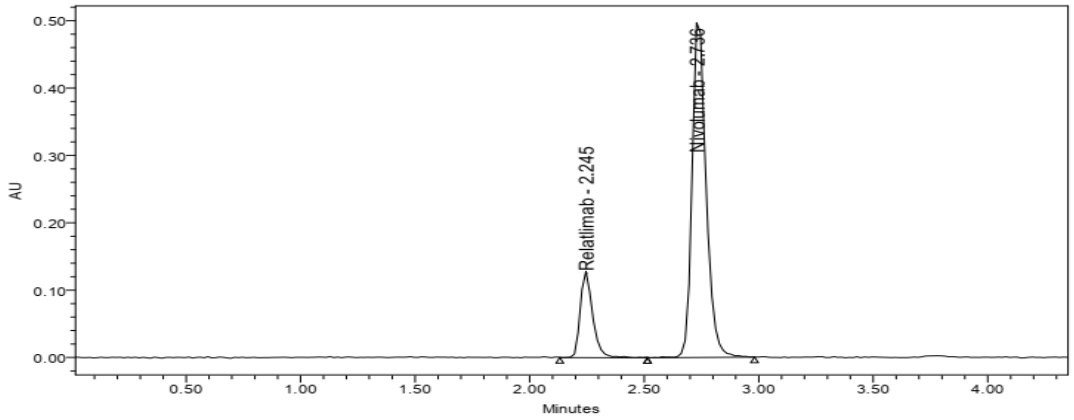

Figure 3. Optimized chromatogram

Table 2: System suitability results

S no	Relatlimab			Nivolumab			
Inj	RT (min)	USP Plate Count	Tailing	RT (min)	USP Plate Count	Tailing	RS
1	2.248	7753	1.28	2.737	9451	1.19	4.5
2	2.250	7471	1.25	2.747	9220	1.16	4.5
3	2.252	7848	1.28	2.751	9662	1.16	4.5
4	2.253	7430	1.27	2.752	9435	1.19	4.7
5	2.259	7417	1.28	2.757	9322	1.18	4.5
6	2.262	7940	1.27	2.759	9675	1.20	4.0

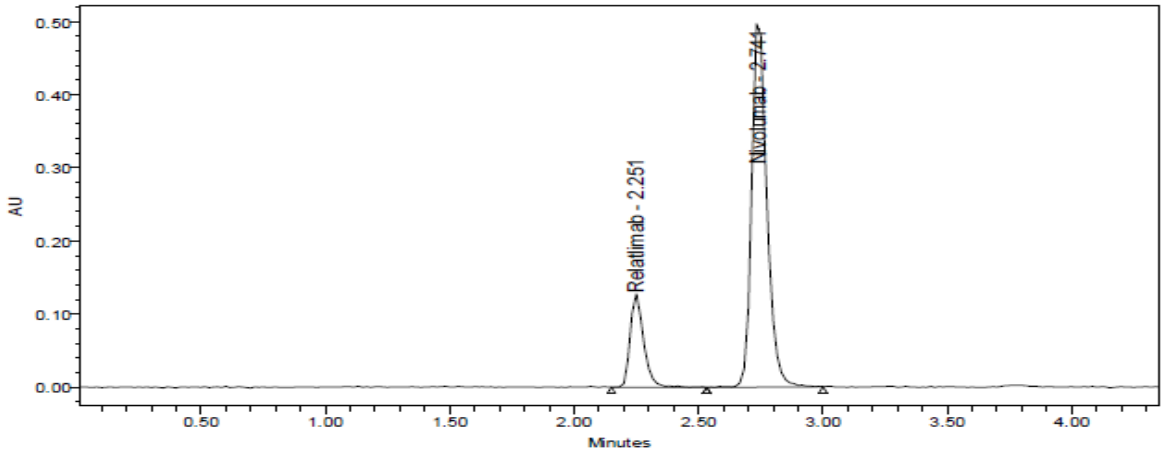

Figure 4. System suitability Chromatogram

Table 3: Specificity data

Sample name	Retention time(mins)	Area
Relatlimab	2.245	1268571
Nivolumab	2.736	3545246

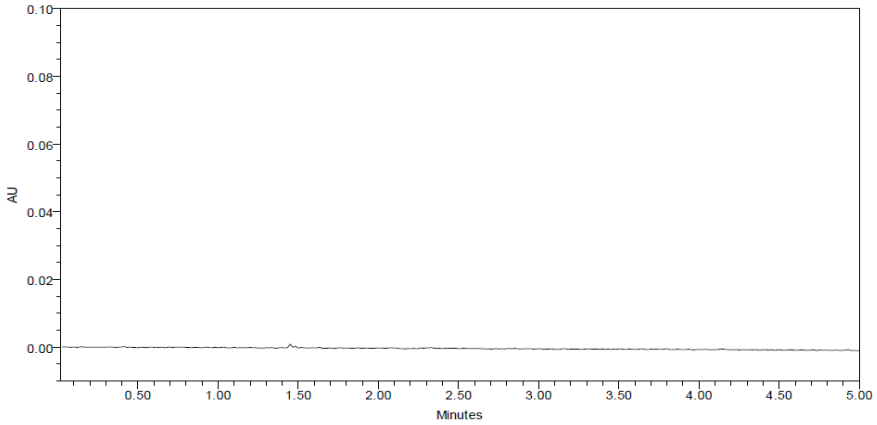

Figure 5. Specificity of Nivolumab and Relatlimab

Linearity:

Calibration data is given in table and regression data in table 4 and calibration curve in figure.

Table 4: Calibration data of Nivolumab and Relatlimab

Relatlimab		Nivolumab	
Conc (µg/mL)	**Peak area**	**Conc (µg/mL)**	**Peak area**
0	0	0	0
12	904502	4	316420
24	1937066	8	635671
36	2769191	12	947825
48	3648778	16	1262424
60	4631236	20	1571849
72	5516812	24	1872519

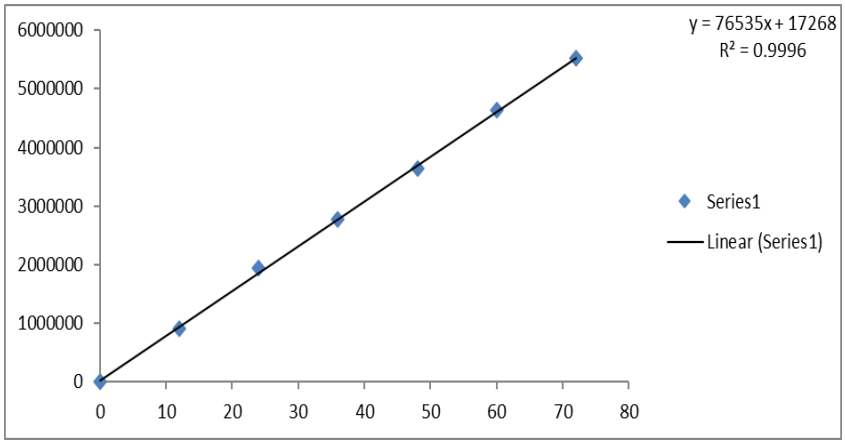

Figure.6 Calibration curve of Nivolumab

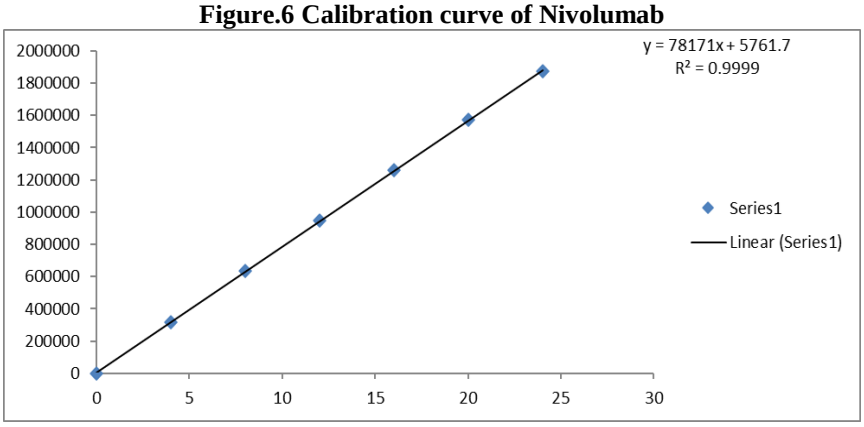

Figure 7. Calibration curve of Relatlimab

Table 5: regression data

Parameter	Relatlimab	Nivolumab
Conc range (µg/mL)	4 - 24	12-72
Regression Equation	y = 78171x + 5761.7	y = 76535x + 17268
Co-relation	0.9999	0.9996

Accuracy:
Recovery data

Table 6: recovery data of Nivolumab and Relatlimab

	Relatlimab			Nivolumab		
% Level	**gained (µg/mL)**	**recovered (µg/mL)**	**% Recovery**	**gained (µg/mL)**	**recovered (µg/mL)**	**% Recovery**
50%	8	7.95	99.39	24	23.85	99.36
	8	7.96	99.48	24	23.84	99.33
	8	7.97	99.64	24	23.78	99.07
100%	16	15.95	99.67	48	47.55	99.07
	16	16.01	100.08	48	47.78	99.55
	16	15.96	99.72	48	47.93	99.84
150%	24	23.96	99.82	72	71.32	99.05
	24	23.91	99.63	72	71.45	99.24
	24	23.94	99.73	72	71.51	99.32
Mean % recovery	99.68			99.32		

System precision was performed and the data was shown in table.

Table 7: System precision of Nivolumab and Relatlimab

S. No	Area of Nivolumab	Area of Relatlimab
1.	3596053	1259500
2.	3611613	1268205
3.	3562224	1267912
4.	3583850	1268893
5.	3584551	1259588
6.	3567752	1265401
Mean	**3584341**	**1264917**
S.D	**18141.5**	**4325.9**
%RSD	**0.5**	**0.3**

The % RSD for the peak areas of Nivolumab and Relatlimab obtained from six replicate injections of standard solution was within the limit.

Method Precision: The precision of the method was determined by analyzing a sample of Nivolumab and Relatlimab and shown in table.

Table 8: method Precision

S. No	Area of Nivolumab	Area of Relatlimab
1.	3588346	1262762
2.	3588711	1263892
3.	3586845	1267252
4.	3565861	1269414
5.	3556485	1263880
6.	3563766	1262737
Mean	**3575002**	**1264990**
S.D	**14552.9**	**2726.4**
%RSD	**0.4**	**0.2**

From the above results, the % RSD of method precision study was within the limit for Nivolumab and Relatlimab.

Robustness: Robustness conditions like Flow minus (0.9ml/min), Flow plus (1.1ml/min), mobile phase minus (55B:45A), mobile phase plus (65B:35A), temperature minus (27°C) and temperature plus(33°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.

Table 9: Robustness data for Nivolumab and Relatlimab.

Condition	%RSD of Nivolumab	%RSD of Relatlimab
Flow rate (-) 0.9ml/min	0.3	0.3
Flow rate (+) 1.1ml/min	0.4	0.4
Mobile phase (-) 55B:45A	0.3	0.2
Mobile phase (+) 65B:35A	0.2	0.3
Temperature (-) 27°C	0.6	0.1
Temperature (+) 33°C	0.4	0.2

Sensitivity:

Table 10: sensitivity of Nivolumab and Relatlimab.

Molecule	LOD	LOQ
Nivolumab	0.08	0.02
Relatlimab	0.25	0.06

Force Degradation Studies: shows degradation conditions and table shows the obtained degraded data and purity plot chromatogram in figure.

Table 11: degradation conditions

Stress condition	Solvent	Temp(^{0}C)	Exposed time
Acid	2N HCL	60^0c	30 mins
Base	2N NAOH	60^0c	30 mins
Oxdation	20% H_2O_2	60^0c	30 mins
Thermal	Diluent	105^0c	6 hours
Photolytic	Diluent	-	-
Hydrolytic	Water	60^0c	

Table 12: degradation data

Type of degradation	Nivolumab			Relatlimab		
	area	%recovered	% degraded	area	%recovered	% degraded
Acid	3403766	94.87	5.13	1182252	93.37	6.63
Base	3428711	95.56	4.44	1189880	93.97	6.03
Peroxide	3456053	96.32	3.68	1199176	94.71	5.29
Thermal	3551306	98.98	1.02	1232236	97.32	2.68
Uv	3551594	98.99	1.01	1241745	98.07	1.93
Water	3564912	99.36	0.64	1256578	99.24	0.76

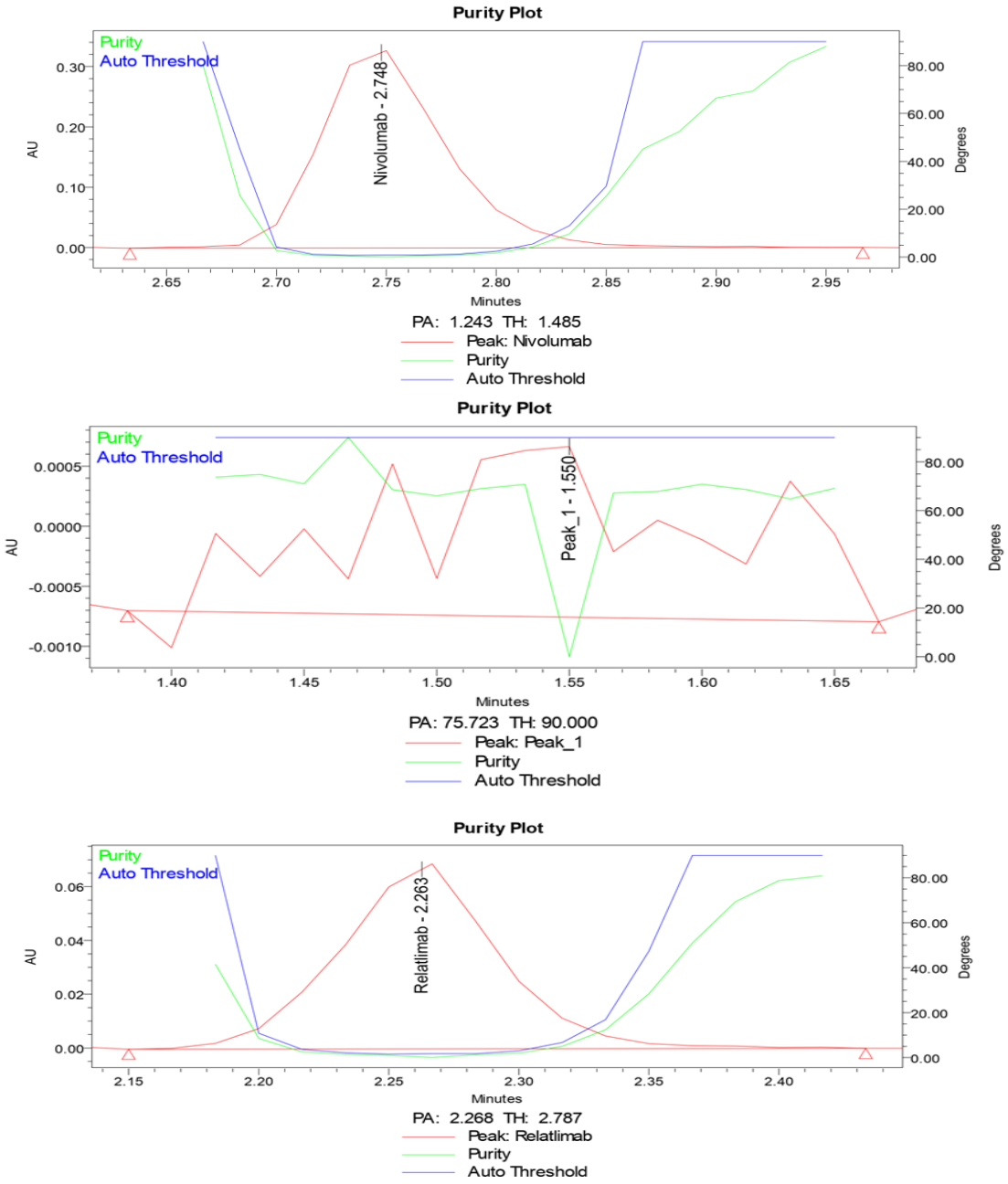

Figure 8. Purity plots for Acid Condition for Nivolumab and Relatlimab.

Assay: Opdualag injection, bearing the label claim Nivolumab 240mg, Relatlimab 80mg. Assay was performed with the above formulation. Average % Assay for Nivolumab and Relatlimab obtained was 99.64% and 99.91% respectively.

Table 11: assay data

	Nivolumab			Relatlimab		
S.no	**Std Area**	**Sample area**	**% Assay**	**Std Area**	**Sample area**	**% Assay**
1	3596053	3588346	100.01	1259500	1262762	99.73
2	3611613	3588711	100.02	1268205	1263892	99.82
3	3562224	3586845	99.97	1267912	1267252	100.08
4	3583850	3565861	99.38	1268893	1269414	100.26
5	3584551	3556485	99.12	1259588	1263880	99.82
6	3567752	3563766	99.33	1265401	1262737	99.73
Avg	**3584341**	**3575002**	**99.64**	**1264917**	**1264990**	**99.91**
Stdev	**18141.5**	**14552.9**	**0.406**	**4325.9**	**2726.4**	**0.22**
%RSD	**0.5**	**0.4**	**0.4**	**0.3**	**0.2**	**0.2**

Assay was calculated by the formula:

		AT	WS	1	100	10	P	FV		
	% Assay = --------X--------X-------X------- X -------X --------X ----------								X 100	
		AS	100	10	1	1	100	L.C		
AT		Average Peak area of sample in test solution								
AS		Mean peak area of sample in standard solution								
WS		Weight of drug working standard taken in mg								
P		Assay of drug working standard in % on dried basis								
L.C		Label Claim								

Conclusion:

The experiments demonstrated the ease of use, accuracy, and precision of the unique suggested method for simultaneous estimate of relatlimab and nivolumab. It works well because of its excellent resolution, short retention time, and degradant separation. The suggested method is affordable and appropriate for pharmaceutical industry standard evaluations.

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