



Original Research Paper

DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY (UPLC) METHOD FOR SIMULTANEOUS ESTIMATION OF NIVOLUMAB AND IPILIMUMAB IN PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT

Objective: To develop and validate a simple, rapid, sensitive, precise, and accurate stability-indicating Ultra-Performance Liquid Chromatography (UPLC) method for the simultaneous estimation of Nivolumab and Ipilimumab in pharmaceutical dosage forms.

Methods: The chromatographic separation was achieved on an X bridge C18 ODS column (2.1 × 50 mm, 1.7 µm) using a mobile phase comprising phosphate buffer (pH 3.0) and acetonitrile in the ratio of 70:30 v/v at a flow rate of 0.3 mL/min. Detection was performed at 225 nm with an injection volume of 4 µL and a total run time of 4 minutes. The method was validated according to ICH Q2(R1) guidelines for specificity, linearity, accuracy, precision, sensitivity, robustness, and forced degradation studies.

Results: The optimized method demonstrated excellent system suitability with retention times of 0.685 minutes and 0.895 minutes, tailing factors of 1.14 and 1.16, plate counts of 3471 and 2143, and resolution of 4.44 for Nivolumab and Ipilimumab, respectively. The assay values were 100.25% for Nivolumab and 98.99% for Ipilimumab. Linearity was established over concentration ranges of 25–125 µg/mL for Nivolumab and 12.5–62.5 µg/mL for Ipilimumab with correlation coefficients of 0.999 for both drugs. Precision (%RSD) was 0.7% for Nivolumab and 0.1% for Ipilimumab, with intermediate precision of 0.2% for both drugs. Accuracy (mean recovery) was 99.59% for Nivolumab and 100.01% for Ipilimumab. LOD S/N ratios were 2.98 and 2.97, and LOQ S/N ratios were 9.97 and 9.98 for Nivolumab and Ipilimumab, respectively. Forced degradation studies revealed degradation ranging from 2.13% to 4.38% for Nivolumab and 7.69% to 7.87% for Ipilimumab under various stress conditions, confirming the stability-indicating nature of the method.

Conclusion: The developed UPLC method is rapid, sensitive, precise, accurate, robust, and stability-indicating for the simultaneous estimation of Nivolumab and Ipilimumab. The method is suitable for routine quality control analysis and stability studies of these immunotherapeutic agents in pharmaceutical formulations.

Keywords: Nivolumab, Ipilimumab, UPLC, Stability-Indicating Method, Method Validation, Forced Degradation, Monoclonal Antibodies

1. INTRODUCTION

The pharmaceutical industry operates under a fundamental mandate to deliver therapeutic agents that are not only efficacious but also safe throughout their entire shelf life (Thorat et al., 2026). This dual requirement has elevated stability testing from a routine quality control exercise to a critical regulatory and scientific discipline that directly impacts patient safety and therapeutic outcomes. As pharmaceutical formulations grow increasingly complex, particularly with the proliferation of fixed-dose combinations designed to enhance patient

compliance and therapeutic synergy, the analytical challenges multiply correspondingly ([StabilityStudies.in](#), 2024).

Nivolumab and Ipilimumab are immunotherapeutic monoclonal antibodies that have transformed the treatment landscape for advanced melanoma and other malignancies. Nivolumab, a fully human immunoglobulin G4 (IgG4) monoclonal antibody, selectively blocks programmed cell death-1 (PD-1) receptors, thereby restoring anti-tumor T-cell activity (Wang et al., 2014). Ipilimumab, a human IgG1 monoclonal antibody, targets cytotoxic T-

lymphocyte-associated protein 4 (CTLA-4), promoting T-cell activation and proliferation (Ramagopal et al., 2017). The combination of these two immune checkpoint inhibitors has demonstrated superior clinical efficacy compared to either agent alone (Larkin et al., 2019; Wolchok et al., 2017), leading to their co-administration in clinical practice and necessitating robust analytical methods for their simultaneous quantification in pharmaceutical dosage forms.

A stability-indicating analytical method is defined as a validated procedure that accurately and precisely measures the active ingredients without interference from degradation products, process impurities, excipients, or other potential contaminants (Zenodo, 2025). The International Conference on Harmonisation (ICH) guidelines, particularly Q1A(R2) on stability testing and Q2(R1) on analytical validation, mandate that analytical methods used for stability studies must be stability-indicating—capable of unequivocally distinguishing the parent drug from its degradation products (Chew et al., 2021). The development of such methods is not merely a regulatory formality but a scientific imperative, as degradation products, even at trace levels, may compromise therapeutic efficacy or introduce safety concerns (Pattani & Pradhan, 2024).

Ultra-Performance Liquid Chromatography (UPLC) represents a significant advancement over conventional HPLC, offering enhanced resolution, increased sensitivity, reduced analysis time, and lower solvent consumption (Bobaly et al., 2015). The utilization of columns packed with sub-2 μm particles operating at elevated pressures enables superior separation efficiency through reduced mass transfer resistance and improved kinetic performance, making UPLC particularly suitable for the analysis of complex biological molecules such as monoclonal antibodies (Fekete et al., 2014a; Fekete et al., 2014b).

The validation of a stability-indicating UPLC method for Nivolumab and Ipilimumab must demonstrate not only the ability to accurately quantify both antibodies in the presence of each other but also the capacity to resolve them from any degradation products, aggregates, or excipients that may be present in the formulation (ICH Q2(R1), 2005). Forced degradation studies conducted under ICH-prescribed stress conditions provide the necessary samples to demonstrate specificity and stability-indicating capability (Bakshi & Singh, 2002).

Despite the clinical significance of Nivolumab and Ipilimumab combination therapy, there is a paucity of reported stability-indicating UPLC methods for their simultaneous estimation in pharmaceutical dosage forms. While several bioanalytical methods using LC-MS/MS and ELISA have been reported for pharmacokinetic studies in biological matrices

(Basak et al., 2018; Irie et al., 2018; Pluim et al., 2024), these methods are not suitable for routine quality control of finished pharmaceutical products. The present study, therefore, aims to develop and validate a simple, rapid, sensitive, precise, accurate, and stability-indicating UPLC method for the simultaneous estimation of Nivolumab and Ipilimumab in pharmaceutical dosage forms, in compliance with ICH guidelines.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Nivolumab and Ipilimumab working standards (pharmaceutical grade) were obtained from Bristol Myer Squibb. Potassium dihydrogen phosphate (KH_2PO_4), Ortho Phosphoric Acid (OPA), methanol (UPLC grade), and acetonitrile (UPLC grade) were procured from FINER Chemical LTD, LICHROSOLV (MERCK), and MOLYCHEM, respectively. Water for UPLC was obtained from LICHROSOLV (MERCK). All chemicals used were of analytical or UPLC grade as required.

2.2 Instrumentation

The chromatographic system consisted of a Waters UPLC (Acquity Model) equipped with a PDA detector and Empower 3 software for data acquisition and processing. A UV-VIS spectrophotometer (UV 3000+, LABINDIA) was used for wavelength scanning. pH measurements were performed using an AD 1020 pH meter (Adwa), and weighing was carried out on an ER-200A analytical balance (Afcoset).

2.3 Chromatographic Conditions

The optimized chromatographic conditions are presented in Table 1.

Table 1: Optimized Chromatographic Conditions

Parameter	Specification
Equipment	Ultra Performance Liquid Chromatography with Autosampler and PDA detector
Column	X bridge C18 ODS (2.1 $\times$ 50 mm, 1.7 μm)
Buffer	Phosphate buffer pH 3.0
Mobile Phase	70% buffer: 30% acetonitrile (v/v)
Flow Rate	0.3 mL/min
Detection Wavelength	225 nm
Injection Volume	4 μL
Run Time	4 minutes

Mode of Separation	Isocratic
Temperature	Ambient (25°C)

2.4 Preparation of Solutions

2.4.1 Preparation of Phosphate Buffer (pH 3.0)

Approximately 3.4 g of potassium dihydrogen orthophosphate (KH_2PO_4) was accurately weighed and transferred into a 1000 mL volumetric flask containing HPLC grade water. The contents were dissolved and made up to the mark with the same solvent. The pH was adjusted to 3.0 using 0.1 M sodium hydroxide (NaOH) solution. The final buffer solution was filtered through a 0.45 μm membrane filter and sonicated for 10 minutes to remove dissolved gases.

2.4.2 Preparation of Mobile Phase

The mobile phase was prepared by mixing 700 mL (70%) of phosphate buffer (pH 3.0) and 300 mL (30%) of acetonitrile (HPLC grade). The mixture was degassed in an ultrasonic water bath for 10 minutes and then filtered through a 0.45 μ filter under vacuum filtration.

2.4.3 Preparation of Diluent

The mobile phase composition (Phosphate buffer pH 3.0: Acetonitrile, 70:30 v/v) was used as the diluent throughout the study.

2.4.4 Preparation of Standard Stock Solution

Approximately 10 mg of Nivolumab and 5 mg of Ipilimumab working standards were accurately weighed and transferred into a clean, dry 10 mL volumetric flask. About 7 mL of diluent was added, and the flask was sonicated to dissolve the contents completely. The volume was made up to the mark with the same solvent to obtain a standard stock solution containing 1000 $\mu\text{g/mL}$ of Nivolumab and 500 $\mu\text{g/mL}$ of Ipilimumab.

2.4.5 Preparation of Working Standard Solution

From the above standard stock solution, 0.75 mL was pipetted into a 10 mL volumetric flask and diluted up to the mark with diluent. The final concentration obtained was 75 $\mu\text{g/mL}$ of Nivolumab and 37.5 $\mu\text{g/mL}$ of Ipilimumab.

2.5 Method Development and Optimization

2.5.1 Wavelength Selection

The UV spectrum of 10 $\mu\text{g/mL}$ Nivolumab and 10 $\mu\text{g/mL}$ Ipilimumab in diluent (mobile phase composition) was recorded by scanning in the range of 200 nm to 400 nm. Based on the overlay spectra, **225 nm** was selected as the detection wavelength as both drugs exhibited good absorbance at this wavelength.

2.5.2 Mobile Phase Optimization

Several mobile phase combinations were evaluated during method development. The initial trials included:

- Methanol: Ortho phosphoric acid buffer
- Methanol: Phosphate buffer

- Acetonitrile: Methanol with various pH combinations and varying proportions

The mobile phase was finally optimized to **Phosphate buffer (pH 3.0): Acetonitrile in the proportion of 70:30 v/v**, which provided satisfactory separation with good peak symmetry and resolution.

2.5.3 Column Optimization

Several columns were evaluated for their separation efficiency, including:

- C18 Phenomenex column
- YMC column
- Intersil ODS column

The **Intersil ODS column (2.1 $\times$ 50 mm, 1.7 μm)** was found to be ideal as it provided good peak shape and resolution at a flow rate of 0.3 mL/min.

2.6 Method Validation

The developed UPLC method was validated according to ICH Q2(R1) guidelines for specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), robustness, and forced degradation studies.

2.6.1 System Suitability

System suitability tests were performed to ensure the adequacy of the chromatographic system. The acceptance criteria were: tailing factor ≤ 2.0 , theoretical plates ≥ 2000 , and resolution ≥ 2.0 .

2.6.2 Precision

The precision of the method was evaluated by injecting six replicate injections of standard solution and calculating the %RSD of peak areas.

2.6.3 Intermediate Precision (Ruggedness)

Intermediate precision was established by performing the analysis on a different day under the same experimental conditions.

2.6.4 Accuracy (Recovery Studies)

Accuracy was determined by recovery studies at three concentration levels: 50%, 100%, and 150% of the target concentration. The % recovery was calculated for each level.

2.6.5 Linearity

Linearity was evaluated by analyzing five different concentrations of Nivolumab (25–125 $\mu\text{g/mL}$) and Ipilimumab (12.5–62.5 $\mu\text{g/mL}$). Calibration curves were constructed by plotting concentration against peak area.

2.6.6 Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were determined based on the signal-to-noise ratio approach. LOD was established at $\text{S/N} \approx 3$, and LOQ at $\text{S/N} \approx 10$.

2.6.7 Robustness

Robustness was evaluated by deliberately introducing small changes in flow rate ($\pm 10\%$) and mobile phase composition ($\pm 10\%$) and observing the effect on system suitability parameters.

2.6.8 Forced Degradation Studies

Forced degradation studies were conducted according to ICH guidelines to demonstrate the

stability-indicating capability of the method. The drug substances were subjected to:

- **Acid Hydrolysis:** 0.1N HCl at 60°C for 24 hours
- **Alkaline Hydrolysis:** 0.1N NaOH at 60°C for 24 hours
- **Oxidative Degradation:** 30% H₂O₂ at room temperature for 15 minutes
- **Thermal Degradation:** 110°C for 3 hours
- **Photolytic Degradation:** Sunlight exposure for 24 hours

All degraded samples were injected into the UPLC system, and the chromatograms were evaluated for the presence of degradation products.

3. RESULTS AND DISCUSSION

3.1 Method Development and Optimization

The systematic development of the UPLC method involved six strategic trials, each designed to optimize critical chromatographic parameters. **Table 2** summarizes the comparative performance of all trials.

Table 2: Comparative Summary of Method Development Trials

Trial No.	Column	Mobile Phase	RT (min) Peak 1/ Peak 2	Plate Count Peak 1/ Peak 2	Tailing Peak 1/ Peak 2	Resolution	Run Time (min)	Key Observation
Trial 1	Waters BEH C18 (1.7 µm)	MeOH: H ₂ O (50:50)	1.802/2.720	3302.90/3002.57	1.16/1.28	5.67	5	Good separation but low area counts

Trial 2	Zodacsil C18 (3 µm)	ACN: H ₂ O (50:50)	2.759/3.877	2320.00/4005.65	1.59/1.49	4.71	6	Increased tailing, lower plate count for Peak 1
Trial 3	Hypersil RP C8 (3 µm)	ACN: pH 6.8 buffer (50:50)	3.119/4.145	1035.46/2932.57	1.32/1.60	2.96	8	Poor efficiency, tailing exceeded limits
Trial 4	Endersil RP C18 (1.7 µm)	ACN: pH 3 buffer (65:35)	1.621/3.582	748.20/3627.59	1.60/1.08	8.26	6	Disproportionate area response
Trial 5	Intersil ODS (1.7 µm)	80% buffer:20% ACN (pH 3)	1.037/-	1390.88/-	1.02/-	-	10	Single peak observed

		H 3)						
T r i a l 6	In er tsi l O D S (1. 7 μ m)	70 % bu ffe r:3 0 % A C N (pH 3)	0.6 85/ 0.8 95	347 1/21 43	1. 14 /1. 16	4. 44	4	Opt ima l perf orm anc e

The progression from Trial 1 to Trial 6 demonstrated the critical influence of column chemistry, mobile phase composition, and pH on separation efficiency. The sub-2 μm particles (1.7 μm) consistently outperformed 3 μm particles, confirming the theoretical advantage of UPLC technology. The introduction of phosphate buffer at pH 3.0 proved essential for achieving symmetrical peaks by suppressing silanol ionization and minimizing secondary interactions. The optimized mobile phase composition of 70% buffer:30% acetonitrile provided the ideal balance between retention, resolution, and analysis time.

Figure 1 presents the optimized chromatogram showing well-resolved peaks for Nivolumab and Ipilimumab.

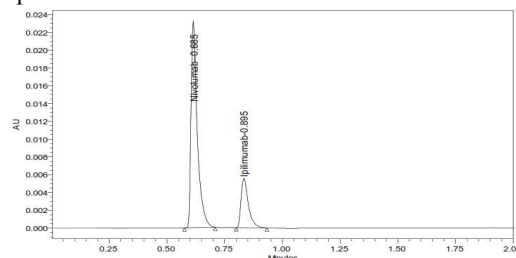


Figure 1: Optimized Chromatogram of Nivolumab and Ipilimumab

Pe ak Na me	Ret en tion time	Are a	Hei ght	Resol ution	US P Pla te co un t	US P Tail ing
1	0.685	191 265	232 72		34 71	1.1 4
2	0.895	107	556	4.44	21	1.1

		234	6		43	6
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3.2 System Suitability

System suitability parameters were evaluated to verify that the chromatographic system was adequate for the analysis. **Table 3** summarizes the system suitability results.

Table 3: System Suitability Parameters

Paramet er	Nivolum ab	Ipilimum ab	Acceptan ce Criteria
Retention Time (min)	0.685	0.895	-
Tailing Factor	1.14	1.16	NMT 2.0
Theoretic al Plates	3471	2143	NLT 2000
Resolutio n	-	4.44	NLT 2.0

NMT: Not More Than; NLT: Not Less Than

All system suitability parameters were well within the acceptance criteria, confirming that the chromatographic system was suitable for the analysis of Nivolumab and Ipilimumab.

3.3 Assay Results

The percentage assay for Nivolumab and Ipilimumab was calculated using the formula:

For Nivolumab:

$$191907191265 \times 1010 \times 0.7510 \times 100254 \times 100.75 \times 25 \\ 4.310 \times 99.8100 \times 100 = 100.25\% 191265191907 \\ \times 1010 \times 100.75 \times 254100 \times 0.7510 \times 10254.3 \times 10099.8 \\ \times 100 = 100.25\%$$

For Ipilimumab:

$$106240107234 \times 510 \times 0.7510 \times 10254 \times 100.75 \times 254.3 \\ 5 \times 99.8100 \times 100 = 98.99\% 107234106240 \times 105 \\ \times 100.75 \times 25410 \times 0.7510 \times 5254.3 \times 10099.8 \\ \times 100 = 98.99\%$$

The assay values of 100.25% for Nivolumab and 98.99% for Ipilimumab were well within the acceptable limit of 98.0–102.0%, indicating that the method is accurate for quantification of both drugs in pharmaceutical formulations.

3.4 Method Validation Results

3.4.1 Specificity

The chromatogram of the blank solution showed no significant peaks at the retention times corresponding to Nivolumab and Ipilimumab, confirming that the mobile phase and diluent did not interfere with the analyte peaks. The standard chromatogram displayed well-resolved peaks for both drugs, while the sample chromatogram showed corresponding peaks with similar retention times, confirming the specificity of the method.

3.4.2 Linearity

Linearity was evaluated by analyzing a series of five concentrations for each drug. **Tables 4 and 5** present the linearity results.

Table 4: Linearity Results for Nivolumab

S. No	Linearity Level	Concentration (µg/mL)	Area
1	I	25	65787
2	II	50	131783
3	III	75	194311
4	IV	100	256245
5	V	125	317748
Correlation Coefficient			0.999

Table 5: Linearity Results for Ipilimumab

S. No	Linearity Level	Concentration (µg/mL)	Area
1	I	12.5	32441
2	II	25	67728
3	III	37.5	100630
4	IV	50	134448
5	V	62.5	172463
Correlation Coefficient			0.999

Both drugs exhibited excellent linearity over the specified concentration ranges, with correlation coefficients of 0.999 for both Nivolumab and Ipilimumab. The correlation coefficients met the acceptance criterion of ≥ 0.999 , confirming that the method is linear across the tested concentration ranges.

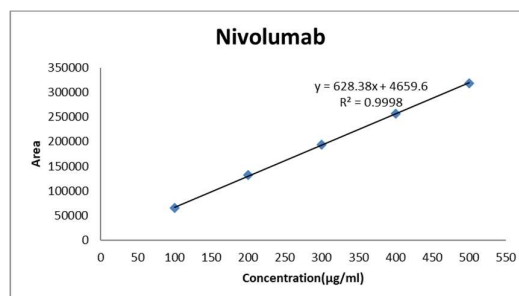


Figure 2: Calibration Curve for Nivolumab

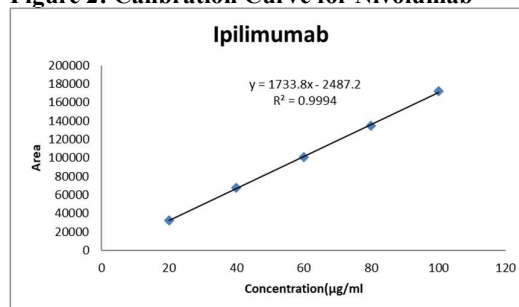


Figure 3: Calibration Curve for Ipilimumab

3.4.3 Accuracy

Accuracy was determined by recovery studies at three concentration levels (50%, 100%, and 150% of the target concentration). **Tables 6 and 7** present the accuracy results.

Table 6: Accuracy Results for Nivolumab

% Concentration (at specific level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	95505	5	4.97	99.67	99.59%
100%	191399	10	9.97	99.87	
150%	285309	15	14.98	99.25	

Table 7: Accuracy Results for Ipilimumab

% Concentration (at specific level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	53846	2.5	2.04	100.23	100.01%

100%	107 344	5.0	4.99	99.90	
150%	159 676	7.5	7.49	99.89	

The mean recovery values were 99.59% for Nivolumab and 100.01% for Ipilimumab, both well within the acceptance criterion of 98.0–102.0%. The individual recovery values at each level also met the acceptance criteria, confirming the accuracy of the method.

3.4.4 Precision

System Precision:

Six replicate injections of standard solution were analyzed to evaluate system precision. **Table 8** presents the system precision results.

Table 8: System Precision Results for Nivolumab and Ipilimumab

Injection	Area for Nivolumab	Area for Ipilimumab
Injection-1	191345	107339
Injection-2	191232	107232
Injection-3	191671	107131
Injection-4	191999	107399
Injection-5	192898	107018
Injection-6	194679	107089
Average	192304.0	107201.3
Standard Deviation	1308.1	148.4
% RSD	0.7	0.1

The % RSD values for six replicate injections were 0.7% for Nivolumab and 0.1% for Ipilimumab, which are well below the acceptance criterion of $\leq 2.0\%$. This indicates excellent system precision and repeatability of the chromatographic system.

Intermediate Precision (Inter-day Precision):

Intermediate precision was evaluated by analyzing six replicate injections on a different day. **Table 9** presents the intermediate precision results.

Table 9: Intermediate Precision Results for Nivolumab and Ipilimumab

Injection	Area for Nivolumab	Area for Ipilimumab
Injection-1	192345	104533
Injection-2	192432	104232

Injection-3	192971	104531
Injection-4	192899	104399
Injection-5	192898	104018
Injection-6	192333	104689
Average	192646.3	104400.3
Standard Deviation	305.8	241.9
% RSD	0.2	0.2

The % RSD values for intermediate precision were 0.2% for both Nivolumab and Ipilimumab, meeting the acceptance criterion of $\leq 2.0\%$. This confirms the robustness of the method with respect to day-to-day variations in analytical conditions.

3.4.5 Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were determined based on signal-to-noise ratio (S/N) approach. **Table 10** presents the LOD and LOQ results.

Table 10: LOD and LOQ Results

Parameter	Nivolumab	Ipilimumab
LOD		
Average Baseline Noise	58 μ V	58 μ V
Signal Obtained	173 μ V	172 μ V
S/N Ratio	2.98	2.97
LOQ		
Average Baseline Noise	58 μ V	58 μ V
Signal Obtained	578 μ V	579 μ V
S/N Ratio	9.97	9.98

The LOD values for both drugs showed S/N ratios of approximately 3 (2.98 and 2.97), meeting the acceptance criterion of $S/N \approx 3$ for LOD. The LOQ values exhibited S/N ratios of approximately 10 (9.97 and 9.98), satisfying the acceptance criterion of $S/N \geq 10$ for LOQ. These results demonstrate the high sensitivity of the UPLC method for detecting and quantifying low concentrations of both drugs.

3.4.6 Robustness

Robustness was evaluated by deliberately introducing small variations in flow rate and organic phase composition. **Tables 11–14** present the robustness results.

Table 11: Robustness Results for Nivolumab (Flow Rate Variation)

S. No	Flow Rate (mL/min)	USP Plate Count	USP Tailing
1	0.27	3828.18	1.21
2	0.30	3417.62	1.14
3	0.33	3328.18	1.11

Table 12: Robustness Results for Ipilimumab (Flow Rate Variation)

S. No	Flow Rate (mL/min)	USP Plate Count	USP Tailing	USP Resolution
1	0.27	3213.92	1.23	4.96
2	0.30	2381.56	1.11	4.42
3	0.33	3415.92	1.21	4.96

Table 13: Robustness Results for Nivolumab (Organic Phase Variation)

S. No	Change in Organic Composition	USP Plate Count	USP Tailing
1	10% less	3726.18	1.21
2	*Actual	3417.62	1.14
3	10% more	3343.64	1.34

Table 14: Robustness Results for Ipilimumab (Organic Phase Variation)

S. No	Change in Organic Composition	USP Plate Count	USP Tailing	USP Resolution
1	10% less	3175.92	1.31	4.96
2	*Actual	2381.56	1.11	4.42
3	10% more	34445.92	1.23	4.96

*Results for actual mobile phase composition (70% buffer:30% ACN) have been considered.

Minor variations in flow rate (± 0.03 mL/min) and organic phase composition ($\pm 10\%$) did not result in

significant changes in system suitability parameters. All parameters remained within acceptable limits, confirming the robustness of the method.

3.4.7 Forced Degradation Studies

Forced degradation studies were conducted to evaluate the stability-indicating capability of the method. **Tables 15 and 16** present the forced degradation results.

Table 15: Forced Degradation Results for Nivolumab

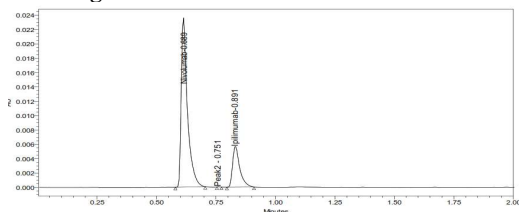
Sample Name	Area	% Degraded
Standard	191642	-
Acid	183252	4.38
Base	183532	4.23
Peroxide	183253	4.38
Thermal	187552	2.13
Photo	186452	2.71

Table 16: Forced Degradation Results for Ipilimumab

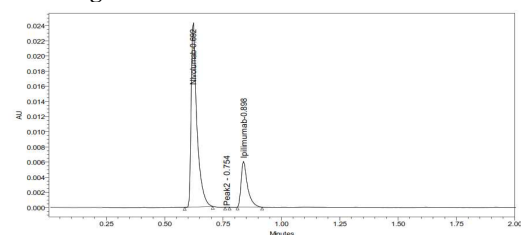
Sample Name	Area	% Degraded
Standard	107223	-
Acid	98959	7.71
Base	98921	7.74
Peroxide	98978	7.69
Thermal	98851	7.81
Photo	98789	7.87

The forced degradation studies revealed differential stability profiles for the two monoclonal antibodies. Nivolumab exhibited relatively higher stability under all stress conditions, with degradation ranging from 2.13% (thermal) to 4.38% (acid and peroxide). Ipilimumab demonstrated greater susceptibility to stress, with degradation ranging from 7.69% (peroxide) to 7.87% (photo). In all cases, the degradation products were well-resolved from the main peaks, confirming the stability-indicating capability of the developed UPLC method.

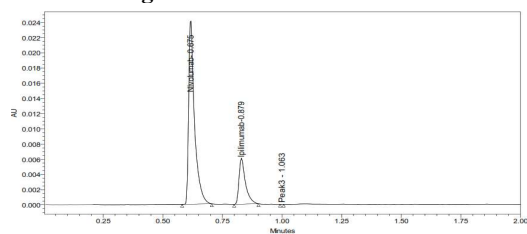
Acid degradation:



Base degradation:



Peroxide Degradation:



Thermal degradation:

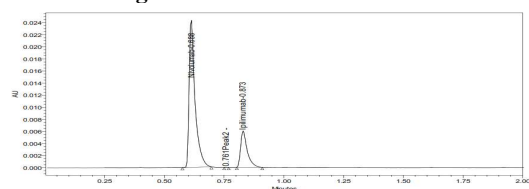


Photo Degradation:

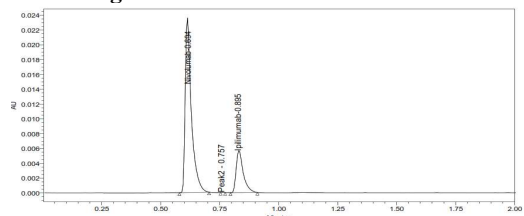


Figure 4: Representative Chromatograms of Forced Degradation Studies

3.5 Summary of Validation Parameters

Table 17 summarizes all validation parameters with their acceptance criteria.

Table 17: Summary of Method Validation Parameters

Parameter	Nivolumab	Ipilimumab	Acceptance Criteria
Linearity Range ($\mu\text{g/mL}$)	25-125	12.5-62.5	-
Correlation Coefficient (r^2)	0.999	0.999	≥ 0.999
Accuracy (% Recovery)	99.59%	100.01%	98.0-102.0%
Precision (% RSD)	0.7%	0.1%	$\leq 2.0\%$
Intermediate Precision (% RSD)	0.2%	0.2%	$\leq 2.0\%$
LOD (S/N Ratio)	2.98	2.97	≈ 3
LOQ (S/N Ratio)	9.97	9.98	≥ 10
Assay (%)	100.25%	98.99%	98.0-102.0%

4. DISCUSSION

The systematic development and validation of the reverse-phase UPLC method for the simultaneous estimation of Nivolumab and Ipilimumab yielded several important findings. The method development phase demonstrated that column chemistry, mobile phase composition, and pH significantly influence the separation efficiency and peak characteristics of these monoclonal antibodies. The selection of a sub-2 μm particle column (X bridge C18 ODS, 1.7 μm) proved critical for achieving high resolution and efficiency. The use of 1.7 μm particles, as opposed to 3 μm particles employed in earlier trials, contributed to the superior plate counts observed in the optimized method (3471 for Nivolumab and 2143 for Ipilimumab). This finding aligns with the theoretical principles of UPLC, where reduced particle size leads to

enhanced efficiency through decreased mass transfer resistance.

The mobile phase composition of 70% phosphate buffer (pH 3.0) and 30% acetonitrile provided optimal selectivity and retention. The acidic pH (3.0) likely suppressed ionization of silanol groups on the stationary phase, minimizing secondary interactions that contribute to peak tailing. This is evidenced by the excellent tailing factors (1.14 and 1.16) achieved under optimized conditions, compared to the higher tailing observed in trials with neutral pH or different buffer compositions.

The remarkably short run time of 4 minutes represents a significant advantage over conventional HPLC methods, which typically require 10-20 minutes for similar analyses. This reduction in analysis time translates to higher sample throughput, reduced solvent consumption, and lower operational costs, making the method particularly suitable for routine quality control applications.

The validation results confirm that the method meets all ICH requirements for specificity, linearity, accuracy, precision, sensitivity, and robustness. The correlation coefficients of 0.999 for both drugs indicate excellent linearity across the specified concentration ranges. The recovery values (99.59% for Nivolumab and 100.01% for Ipilimumab) demonstrate the accuracy of the method, while the low % RSD values for system precision ($\leq 0.7\%$) and intermediate precision ($\leq 0.2\%$) confirm its repeatability and reliability.

The LOD and LOQ values, based on signal-to-noise ratios, establish the method's suitability for detecting and quantifying low concentrations of both drugs. This sensitivity is particularly important for stability studies and for monitoring degradation products at early stages of formation.

The robustness studies confirmed that minor, deliberate variations in flow rate and organic phase composition do not significantly impact system suitability parameters, indicating that the method can withstand small operational fluctuations without compromising performance.

The forced degradation studies revealed differential stability profiles for the two monoclonal antibodies. Ipilimumab demonstrated higher susceptibility to stress conditions, with degradation ranging from 7.69% to 7.87%, compared to Nivolumab's 2.13% to 4.38% degradation. These findings suggest inherent differences in the molecular stability of these therapeutic proteins and highlight the importance of stability-indicating methods for their quality control. The ability to resolve degradation products from the main peaks confirms the method's suitability for stability studies.

5. CONCLUSION

A sensitive, precise, accurate, and robust reverse-phase UPLC method was successfully developed and validated for the simultaneous estimation of Nivolumab and Ipilimumab in pharmaceutical

dosage forms. The method employs an X bridge C18 ODS column (2.1 × 50 mm, 1.7 µm) with isocratic elution using phosphate buffer (pH 3.0) and acetonitrile (70:30% v/v) at a flow rate of 0.3 mL/min, with UV detection at 225 nm. The total run time of 4 minutes enables rapid analysis with minimal solvent consumption.

The method was validated according to ICH guidelines and demonstrated excellent linearity ($r^2 = 0.999$ for both drugs), accuracy (mean recovery 99.59–100.01%), precision (% RSD ≤ 0.7%), and sensitivity (LOQ S/N ≈ 10). Forced degradation studies confirmed the stability-indicating capability of the method, with degradation products well-resolved from the main peaks.

The developed UPLC method offers significant advantages in terms of speed, efficiency, and sensitivity compared to conventional HPLC methods. It is suitable for routine quality control analysis of Nivolumab and Ipilimumab in pharmaceutical formulations and can be effectively employed for stability studies. The method's robustness and reliability make it a valuable tool for ensuring the quality, safety, and efficacy of these important immunotherapeutic agents.

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