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A new RP- HPLC Method Development and Validation for the Concurrent Identification of Nivolumab and Hyaluronidase in Bulk and Pharmaceutical Dosage Form

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A B S T R A C T

A robust HPLC method was developed and validated for the simultaneous estimation of Nivolumab and Hyaluronidase using an Inspire C18 column (250×4.6 mm, 5µm) with a 1.0 mL/min flow rate. The method achieved clear separation with retention times of 2.817 min (Nivolumab) and 3.731 min (Hyaluronidase). System suitability, linearity (R^2> 0.999), precision (%RSD low), and accuracy (98–102% recovery) met validation standards. The method demonstrated good ruggedness, robustness, and sensitivity, with LOD/LOQ values of 1.24µg/mL and 0.04µg/mL, respectively. Intermediate precision confirmed reproducibility across systems. This validated method is suitable for routine quality control and regulatory compliance in pharmaceutical formulations.

Keywords: Nivolumab, Hyaluronidase, Chromatographic Optimization, HPLC, Retention Time, System Suitability, Linearity, Accuracy, Precision, Robustness, Pharmaceutical Analysis, Quality Control.

A R T I C L E I N F O

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C o n t e n t s

1. Introduction

High-Performance Liquid Chromatography (HPLC) is a chemistry-based analytical tool used for quantifying and analyzing mixtures of chemical compounds by separating components based on their distribution between a stationary phase (a packed column with fine particles) and a mobile phase (a liquid like water or alcohol). The technique operates under high pressure (around 6000 psi) due to the use of small particle sizes, enhancing separation efficiency. The basic principle involves injecting a sample into the mobile phase, where compounds migrate at different speeds depending on their affinity for the stationary phase, leading to separation. Each component elutes at a distinct retention time and is detected, generating a chromatogram for qualitative and quantitative analysis. HPLC is widely used in pharmaceutical research for method development and validation, ensuring accuracy, precision, and reliability in drug analysis. Key validation parameters include system suitability (evaluating resolution, reproducibility, and tailing factors), accuracy, precision, linearity, limit of detection (LOD), limit of quantification (LOQ), and robustness. Stability-indicating methods (SIMs) are critical for monitoring drug degradation under stress conditions, ensuring safety and efficacy. The technique evolved from early chromatography methods, with advancements like reversed-phase HPLC and UV detection becoming standard. Method validation confirms that analytical procedures are fit for purpose, supporting drug development, quality control, and regulatory compliance.

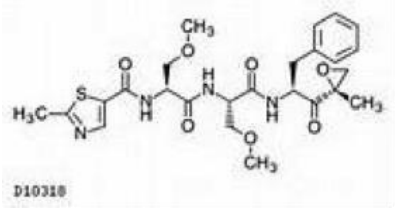

Fig.1. Nivolumab

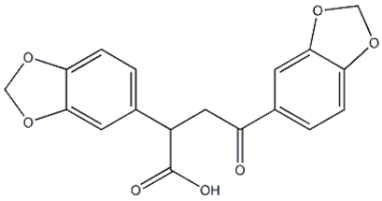

Fig.2. Hyaluronidase

2. Materials and Methods

List of Proposed Materials:

The estimation of Nivolumab and Hyaluronidase was carried out using high-purity HPLC-grade chemicals and reagents. Orthophosphoric acid and triethylamine, both sourced from Qualigens, were specifically used for the analysis of Nivolumab and Hyaluronidase. HPLC-grade water and acetonitrile, also from Qualigens, were used as solvents for all drugs throughout the study. Additionally, HPLC-grade methanol obtained from Rankem was employed as a general solvent in the chromatographic process. All reagents were chosen to ensure the accuracy, reliability, and consistency of the analytical method.

Equipments and instruments used in the study:

The equipment used for the analysis included an electronic balance (model SAB2032) from Scaletec for accurate weighing, and an ultra-sonicator (SE60US) from Labman Scientific India for proper mixing of samples. A thermal oven (i-THERM A17782) from Dwaraka Scientific was used for drying purposes, while pH measurements were taken using a pH meter (ORION STAR A111) from ThermoScientific. Filtration was done using 0.45-micron filter paper from Millipore to remove any particulates. The HPLC analysis was carried out using a WATERS 2690 Separation Module, ensuring precise estimation of Nivolumab and Hyaluronidase.

Method Development

Wave length selection:

UV spectrum of 20µg/ml **Nivolumab** and **Hyaluronidase** in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm and the isobestic λmax of both the drugs obtained at 255 nm.

Optimization of Column:

Inspire C18 Column, (250×4.6mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1.0 ml/min flow.

Optimization of Chromatographic Conditions

Optimized Chromatographic Conditions

The analysis was performed using a High-Performance Liquid Chromatography (HPLC) system equipped with an auto sampler and a DAD or UV detector. The separation was carried out at ambient temperature using an Inspire C18 column (250 × 4.6 mm, 5 µm). The mobile phase consisted of 45% 0.1% orthophosphoric acid buffer (pH 3.5) and 55% acetonitrile, delivered at a flow rate of 1.0 mL/min. The detection wavelength was set at 255 nm, with an injection volume of 10 µL. The total run time for each sample was 10 minutes, ensuring adequate separation and detection of the analytes.

Preparation of buffer and mobile phase:

Preparation of 0.1% orthophosphoric acid buffer (pH 3.5): Prepare 0.1% orthophosphoric acid buffer solution, by adding 1 ml of orthophosphoric acid in 1000 ml water and adjust the solution pH to correctly pH 3.5 by using sodium hydroxide.

Preparation of mobile phase:

Mix a mixture of above Acetonitrile 550ml (55%), 450 ml 0.1% orthophosphoric acid (45%) and degas in ultrasonic water bath for 5 minutes. Filter through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

Acetonitrile: 0.1% OPA (550:450 ml) ratio.

Assay:

Standard Solution Preparation:

Accurately weigh and transfer 200 mg of Nivolumab Working standard into 50 ml clean dry volumetric flask and 10mg Hyaluronidase Working standard into a 200 ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 9ml from the Nivolumab and 0.3ml from the Hyaluronidase above stock solutions into a 25ml volumetric flasks and dilute up to the mark with Diluents. (1440ppm Nivolumab & 0.6ppm Hyaluronidase)

Sample Solution Preparation:

Accurately taken a volume of injection equivalent to 200 mg of Nivolumab into 50 ml clean dry volumetric flask and 10mg Hyaluronidase into a 200 ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 9ml from the Nivolumab and 0.3ml from the Hyaluronidase above stock solutions into a 25ml volumetric flasks and dilute up to the mark with Diluents. (1440ppm Nivolumab & 0.6ppm Hyaluronidase)

Procedure: Inject 20 µL of the standard, sample into the chromatographic system and measure the areas for the Nivolumab and Hyaluronidase peaks and calculate the % Assay by using the formulae.

3. Results and Discussion

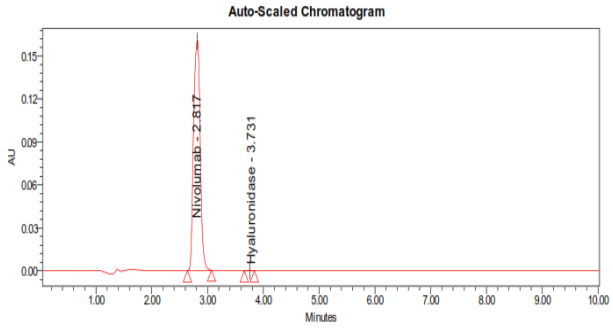

Fig.3. Optimized chromatogram

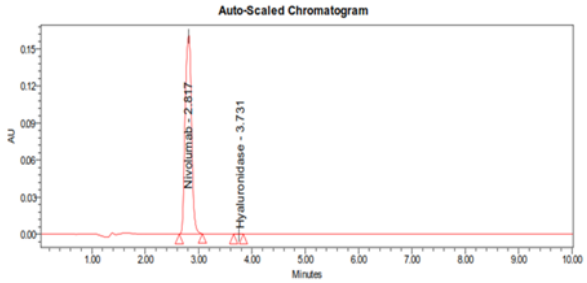

Figure 4: Chromatogram for system suitability

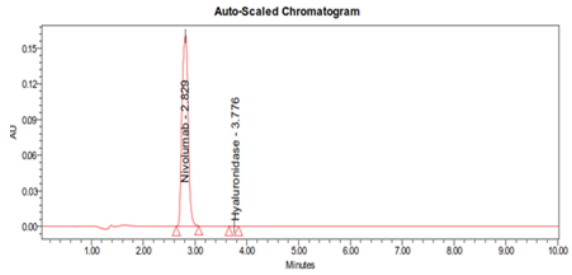

Figure 6: Chromatogram for Standard

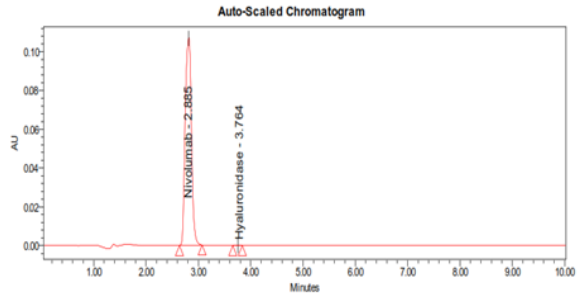

Figure 7: Chromatogram for Sample

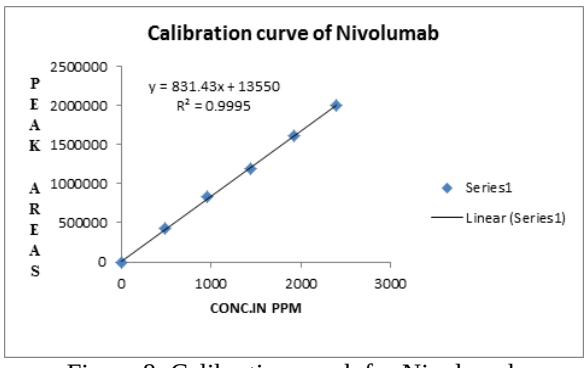

Figure 8: Calibration graph for Nivolumab

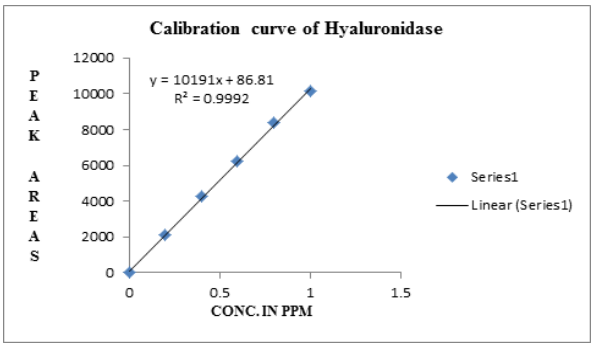

Figure 9: Calibration graph for Hyaluronidase

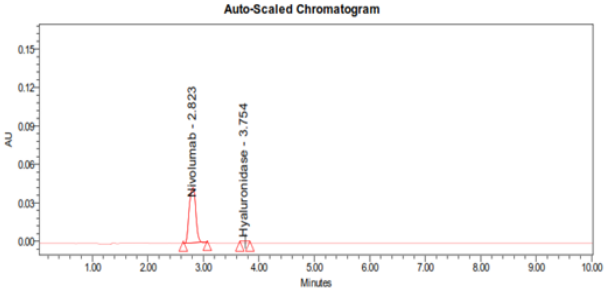

Figure 10: Chromatogram of Nivolumab and Hyaluronidase showing LOD

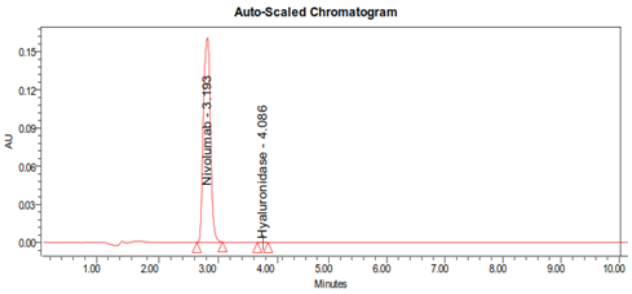

Figure 11: Chromatogram showing less flow

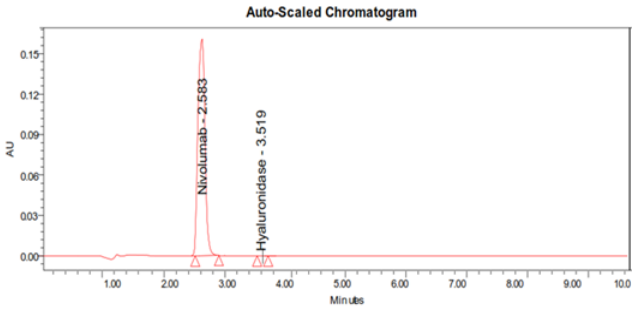

Figure 12: Chromatogram showing more flow

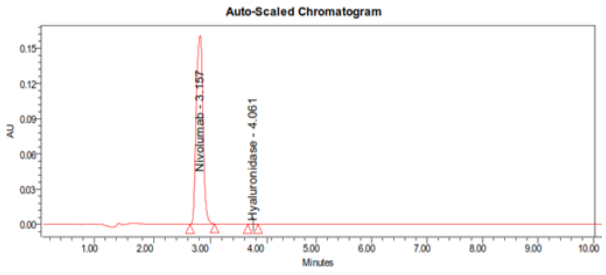

Figure 54: Chromatogram showing less organic composition

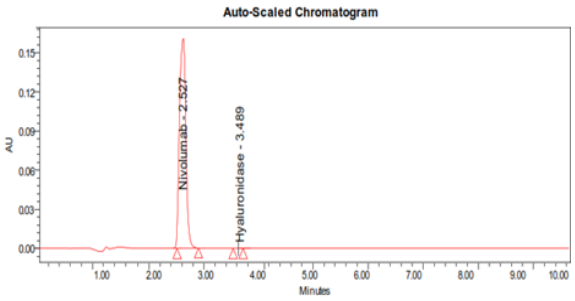

Figure 55: Chromatogram showing more organic composition

Table 1: Results for Standard

Name(STD)	RT(min)	Area(µV sec)	Resolution	USP tailing	USP plate count
Nivolumab	2.829	1185268	4.3	1.24	4112
Hyaluronidas	3.776	6378		1.30	5998

Table 2: Results for Sample

Name (Sample)	RT(min)	Area(µV sec)	Resolution	USP tailing	USP plate count
Nivolumab	2.885	1052551	4.5	1.36	4987
Hyaluronidase	3.764	6215		1.45	5989

Table 3: Results of Assay for Nivolumab and Hyaluronidase

Drug names	Label Claim (mg)	% Assay
Nivolumab	120mg	98.90
Hyaluronidase	0.05mg	99.20

Table 4: Analytical performance parameters of Nivolumab and Hyaluronidase

Parameters	Nivolumab	Hyaluronidase
Slope (m)	83143	10191
Intercept (c)	13550	86.81
Correlation coefficient (R^2)	0.9995	0.9992

Table 5: Results of Precision for Nivolumab and Hyaluronidase

Injection	Area of Nivolumab	Area of Hyaluronidase
Injection-1	1150468	6523
Injection-2	1106257	6541
Injection-3	1103202	6537
Injection-4	1101046	6589
Injection-5	1101202	6572
Injection-6	1103465	6539
Average	1110940	6550.166667
Standard Deviation	17761.68827	22.74801579
%RSD	1.6	0.3

Table 6: Results of Intermediate precision for Nivolumab and Hyaluronidase

Injection	Area of Nivolumab	Area of Hyaluronidase
Injection-1	1103654	6413
Injection-2	1126728	6425
Injection-3	1129547	6430
Injection-4	1128745	6437
Injection-5	1129764	6491
Injection-6	1126452	6420
Average	1124148.333	6436
Standard Deviation	9253.737239	25.71640203
%RSD	0.8	0.4

Table 7: Results of LOD

Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio	Conc. In ppm
Nivolumab	54	156	2.8	1.24 µg/ml
Hyaluronidase	54	150	2.7	0.04 µg/ml

Table 8: Results of LOQ

Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio	Conc. In ppm
Nivolumab	54	539	9.98	4.30 µg/ml
Hyaluronidase	54	500	9.25	0.14 µg/ml

4. Conclusion

In conclusion, the validated HPLC method for Nivolumab and Hyaluronidase is precise, accurate, linear, sensitive, and robust, making it highly reliable for routine pharmaceutical analysis and quality control applications. The method complies with regulatory standards, ensuring accurate identification and quantification of these drugs. Given its high reproducibility and stability, this analytical approach is well-suited for effective quality assurance and validation of Nivolumab and Hyaluronidase formulations.

Conflict of Interest

We declare that we have no conflict of interest

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