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Original Research Article

RP-HPLC Method Development and Validation for Estimation of Nivolumab in Marketed Formulations

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Abstract:
A simple and precise RP-HPLC method for the estimation of Nivolumab in marketed dosage forms was developed and validated. The chromatographic separation of the drug was done with a Waters Sun Fire C8 Column (250 mm × 4.6 mm i.d., particle size 5 µ) using 0.3% TFA, acetonitrile and methanol (45:35:20 v/v/v) as a mobile phase. The instrument was set at flow rate of 1.2 $mLmin^{-1}$ at ambient temperature and the wavelength of UV-visible detector was 230nm. The method showed excellent linearity over a range of 10-250 μgmL^{-1} for the drug. The correlation coefficient for Nivolumab was noted to be 0.9998. The mean recovery values were found to be 99.38% and 99.90%. The results suggest that the proposed method could be suitable for quantitative determination of Nivolumab in pharmaceutical preparations and also for quality control in bulk manufacturing. The *f*-test and *t*-test at 95% confidence level were applied on data for statistical analysis.

Introduction

Nivolumab (NB) is a genetically engineered, fully human immunoglobulinG4 (IgG4) monoclonal antibody specific for human PD-1. The IgG4 isotype was engineered to obviate antibody-dependent cellular cytotoxicity (ADCC). Most monoclonal antibodies in therapeutic oncology contain the IgG1 subtype, which have the most significant ADCC whereas IgG4 subtype possesses minimal ADCC activity. An intact ADCC has the potential to deplete activated T cells and tumor-infiltrating lymphocytes and diminish activity as PD-1 is expressed on T effector cells and other immune cells. Nivolumab was first PD-1 immune checkpoint inhibitor to be approved for use in advanced, squamous (SQ) nonsmall cell lung cancer (NSCLC). Furthermore, Nivolumab is one of the most extensively studied immune checkpoint inhibitors across various tumor types and has anticancer activity against several tumor types, including melanoma, NSCLC, and renal cell cancer (RCC). Nivolumab monotherapy presents a favorable benefit-risk profile in patients with previously treated advanced or metastatic NSCLC as well as in patients with advanced or metastatic RCC. Nivolumab is approved in the USA for the treatment of patients with metastatic SQ NSCLC which has shown

progression on or after platinum-based chemotherapy and in the European Union (EU) for the treatment of adults with locally advanced or metastatic SQ NSCLC after prior chemotherapy treatment(1-4). Nivolumab is also approved in the USA and the EU for the treatment of advanced melanoma and in the USA for use in previously treated patients with advanced, non-SQ NSCLC. Several clinical trials are underway for other indications, such as the 1st line in RCC/NSCLC, glioblastoma multiforme, head and neck cancer, small cell lung cancer, gastrointestinal malignancies, and genitourinary malignancies. NB and other immune checkpoint agents, on the other hand, cause a variety of immune-related adverse effects including dermatological, gastrointestinal, endocrine, hepatic, neurological and other effects on other organs. It had not been tested in pregnant women but based on the mechanism of action and animal studies, is probably toxic to the baby, it is not known if it is secreted in breast milk. Side effects include severe immune-related inflammation of the lungs, colon, liver, kidneys, and thyroid, and there are effects on skin, central nervous system, the heart, and the digestive system (5-8). The objective of this work was to develop a simple liquid chromatography method, which could serve as an assay for determination of NB in marketed dosage forms. Literature survey reveals number of HPLC based methods is already in existence for evaluation of NB with other drugs in various marketed dosage forms as well as in biological fluids [9-14]. But no HPLC method has been published for the determination of only NB in dosage form. Hence, an attempt was made to develop a simple, precise and accurate method for the estimation of NB in pharmaceutical dosage forms. This research article describes the development and validation of an isocratic reversed phase HPLC method for determination of NB in pharmaceutical dosage forms as per ICH guidelines.

Experimental

Chemicals and Reagents

Methanol and acetonitrile were procured from Thermo Fisher Scientific India Pvt. Ltd. New Delhi, India. Tri fluoro acetic acid (TFA) was procured from Central Drug House (P) Limited, New Delhi, India. Mili Q water was used throughout the study. Other chemicals used in this study were of analytical or HPLC grade.

Instrumentation and Chromatographic Conditions

The analysis was carried out on Waters Alliance e-2695 separating module (Waters Co., MA, USA) using photo diode array detector (waters 2998) with auto sampler and column oven. The instrument was controlled by Empower software (version 6.00.00.00) installed with equipment for data collection and acquisition. Waters Sun Fire C8 Column (250 mm × 4.6 mm i.d., particle size 5 µ), eluted with mobile phase at the flow rate of 1.2 $mLmin^{-1}$ was used. The mobile phase consisted of 0.3% TFA, acetonitrile and methanol (45:35:20 v/v/v). Measurements were made with injection volume 10 µL and UV detection at 230 nm. All analyses were performed at ambient temperature.

Preparation of Standard and Sample Solution

Accurately weighed 100mg of NB (99.87%) was transferred into a 100mL volumetric flask and dissolved in the methanol. Volume was made up to the mark with methanol. A standard solution was prepared from the stock solution by transferring 10mL of stock solution to a 100mL volumetric flask and diluting with methanol to get a solution of 100 μgmL^{-1} of NB. The method was used for estimation of NB in the marketed formulations. For sample preparation, methanol was used as diluent. The formulation equivalent to 100mg of NB was transferred in to 100mL volumetric flask and dissolved in 50mL of methanol. Volume was made up to the mark with diluent. The solution was further diluted with mobile phase to obtain

desired concentration and was subjected to HPLC analysis as described earlier. From the peak area of NB, the amount of drugs in samples was calculated.

Method Validation

The optimized chromatographic conditions were validated by evaluating specificity, range, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), robustness and system suitability parameters in accordance with the ICH guidelines Q2 (R1). To assess the linearity and range of the developed method, six different standard concentrations (10, 25, 50, 125, 200 and 250 μgmL^{-1}) of NB were prepared. The analyses were performed in duplicate. The peak area values were plotted against corresponding concentrations. The accuracy and precision were measured by performing the assay of samples (spiked placebos) prepared at three concentration levels of 50%, 100% and 150% of the standard concentration, with two replicates for each concentration. The % recovery and % relative standard deviation (RSD) were calculated for each of the replicate samples. LOD and LOQ of the method were calculated based on the standard deviation of the response (σ) and slope approach as defined in ICH guidelines. The LOD was calculated using the formula 3.3*σ/slope, and the LOQ was calculated using the formula 10*σ/slope. Robustness of the method was investigated under a variety of conditions including flow rate, column temperature, wavelength and percentage of solvent in the mobile phase (15-18).

Results and Discussion

In this work, a liquid chromatography method for the determination of NB in bulk drug and pharmaceutical formulations with UV detection was developed and validated as per ICH guidelines for analytical method validation (19-21).

Method Development

The main objective of this work was to develop a HPLC method for determination of NB within a short run time along with peak symmetry between 0.90 and 1.10. The stationary and mobile phases play an important role on theoretical plates, peak shape, symmetry and resolution. To obtain symmetrical peak with better resolution and peak purity, various chromatographic conditions were investigated and optimized for the determination of NB; such as mobile phases with different composition, stationary phases with different packing material etc. Finally, the mobile phase containing 0.3%TFA, acetonitrile and methanol in the ratio of 45:35:20v/v/v was selected and found to be optimal with more theoretical plates (≥23542) and short retention time (5.38, below 7 min). Based on the optimal mobile phase, a highly symmetrical and sharp characteristic peak of NB was further obtained on Waters Sun Fire C8 Column (250 mm × 4.6 mm i.d., particle size 5 μ) with 1.2 $mLmin^{-1}$ flow rate. A typical HPLC chromatogram of standard solution of NB is given in Figure 1.

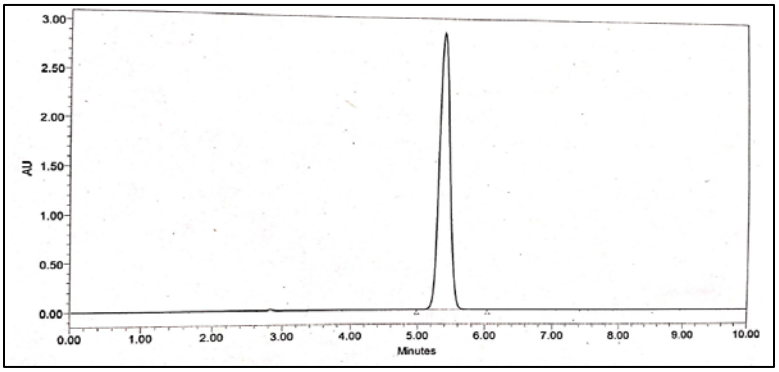

Figure 1: HPLC chromatogram of standard solution of NB

Method Validation

An optimized method must be validated before actual use. System suitability testing was performed as per ICH guidelines for analytical method validation, Q2 (R1). Linear regression data demonstrated an excellent relationship over a concentration range of 10-250 μgmL^{-1} for NB. The linear regression equations for NB was found to be y = 31357x + 24658. The regression coefficient value (r^2) was found to be 0.9998 indicating a high degree of linearity. The linearity curve of NB is given in Figure 2 and linearity parameters for the NB are given Table 1. The specificity studies depicted the complete absence of any other excipients as no peak was reported during the retention time of NB. Standard deviation and slope based method was adopted for determining the LOD and LOQ. The LOD and LOQ of method were found to be 0.5 and 2.0μgmL^{-1}, respectively for NB. The values indicate that the method is sensitive. The lower values of % RSD was found to be 0.51 and 0.62 for intra-day and inter-day precisions respectively indicate (Table 2) that the method is precise. The results showed that the calculated value is less than the critical value, hence there is no significant difference between the results of linearity and precision on three consecutive days. Recovery study was carried out using standard addition method at three different levels of 50%, 100% and 150%. The average % recoveries for NB in marketed formulation were found to be between 99.38 and 99.90 (Table 3). The results revealed that there was no interference. The developed method was successfully applied to analyze NB in marketed formulation. The amounts recovered were expressed as percentage of the label claim. Analysis of marketed formulation was carried out using an optimized mobile phase and HPLC conditions. The average percentage of drug contents obtained by the proposed method for NB was noted to be 99.72% which comply with the official specifications.

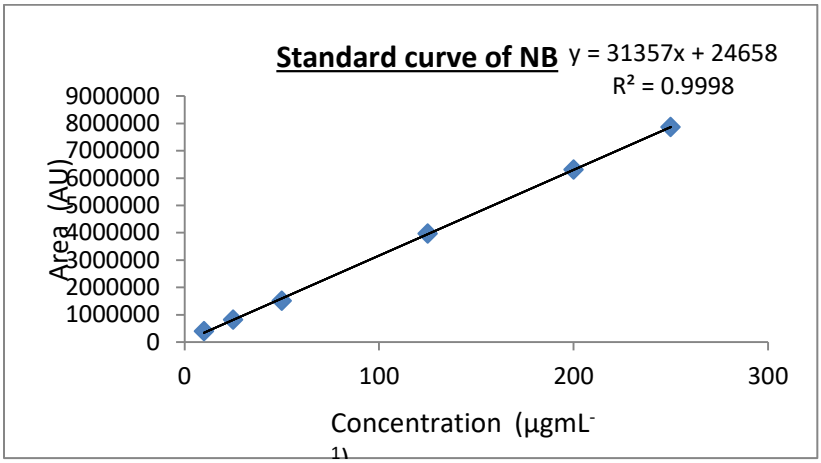

Figure 2: Standard curve of NB

Table 1: Linearity parameters for NB

Linearity Parameter	NB
Range	10-250 μgmL^{-1}
Slope	31357
Intercept	24658
Regression coefficient (R^2)	0.9998
f-test	1.91 (3.85)[a]

Table 2: Statistical treatment of the precision data

Parameter	NB
Intraday or Repeatability (%RSD)	0.51
Inter day (%RSD)	0.62
f-test	3.31 (5.98)[a]
t-test	1.27 (2.64)[a]

Table 3: Percent recovery data NB

% simulated dosage nominal	% Mean	RSD (%)
50	99.53	1.64
100	99.38	1.08
150	99.90	1.77

System Suitability Parameters

For system suitability parameters, six replicates of standard solution of NB were injected. All critical parameters met the acceptance criteria on all days. Parameters such as resolution, tailing factor, theoretical plates, capacity factor, and retention volume of the peaks were calculated. The results are shown in Table 4.

Table 4: System suitability data for NB

Parameters	NB
Retention time (min)	5.38±0.04
Injection precision RSD (%)	1.40
Resolution	-
Tailing factor	0.98
Theoretical plates	23542
Capacity factor	0.73
Retention volume	6.45

Conclusion

A simple isocratic reversed phase HPLC method for estimation of NB was developed and validated as per ICH guidelines. Validation experiments proved that the HPLC method is linear in the proposed working range as well as accurate, precise and specific. The good recovery percentage of marketed forms suggests that the excipients have no interference in the determination. The RSD (%) was also less than 2 showing a high degree of precision of the method. The proposed method was also found to be robust with respect to flow rate, composition of mobile phase, wavelength and column temperature. In addition, simple isocratic elution and easy extraction procedure offered rapid and cost-effective analysis of the drugs. The *f*-test and *t*-test were applied to the data at 95% confidence level, and no statistically significant difference was observed. The proposed method can be used for routine analysis of NB in marketed dosage forms and in the quality control in bulk manufacturing as well.

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