Research Article

Stability Indicating HPLC Method for Simultaneous Determination of Drugs Nivolumab and Ipilimumab

Sharmila Alladi[1*], Konda Ravi Kumar[2] and B. Mallikarjuna[3]

[1]*Department of Pharmacy Jawaharlal Nehru Technological University Kakinada, India*
[2]*Department of Pharmaceutical Chemistry, Hindu College of Pharmacy, India*
[3]*Department of Chemistry, Government College, India*

[*]Address Correspondence to Sharmila Alladi, sharmisssree@gmail.com

Abstract

A simple, accurate, specific and rugged reverse phase liquid chromatographic method was developed for the simultaneous estimation of Nivolumab and Ipilimumab in bulk and tablet dosage form. A RP Inertsil ODS, having dimensions of 150 × 4.6 mm and particle size of 3.5 µ in isocratic mode, with mobile phase containing a mixture of 0.1% orthophosphoric acid and acetonitrile in the proportion (50:50 v/v). The mobile phase was pumped at a flow rate of 1.0 ml/min and the eluent were monitored at 225 nm.

Keywords: Inertsil ODS; Isocratic; Nivolumab and Ipilimumab

Introduction

Nivolumab, marketed as Opdivo, is a medication used to treat cancer [1]. Nivolumab is a monoclonal humanized IgG4 isotype antibody (kappa isotype) approved or under development in many onco-hematological cancers. It targets the programmed cell death-1 (PD-1) receptors of lymphocytes and thereby allowing the immune system to eliminate cancer cells [2]. It is used as a first line treatment for inoperable or metastatic melanoma [3] in combination with ipilimumab [4] if the cancer does not have a mutation [5] in BRAF, as a second line treatment following treatment with ipilimumab and if the cancer has a mutation in BRAF, with a BRAF inhibitor [6], as a second line treatment for squamous non-small lung cancer, and as a second line treatment for renal cell carcinoma. It had not been tested in pregnant women but based on the mechanism of action and animal studies, is probably toxic to the fetus [7]; 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 (Figure 1).

Ipilimumab is the first US FDA approved immune checkpoint blocking antibody drug to harness the patient's own immune cells. One of the post marketing requirements is to develop a cell based neutralizing antibody assay [8]. Here, we share some of the most challenging aspects encountered during the assay development: new cell line construction; an unexpected inhibition of T-cell activation by low concentrations of ipilimumab and two issues caused by sample pretreatment with acid dissociation to overcome drug interference: instability of neutralizing antibody positive control at low pH, and incompatibility of commonly used acid dissociation buffers in the cell assay [9]. After troubleshooting and optimization, we successfully validated the assay and used the assay to test clinical samples to date (Figure 2).
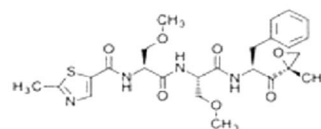
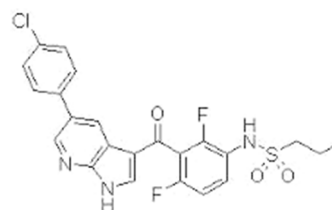

Figure 1: Chemical structure of Nivolumab

Figure 2: Chemical structure of Ipilimumab

Materials and Methods

Instrumentation

To develop a high performance liquid chromatographic method for simultaneous estimation of Nivolumab and Ipilimumab using Shimadzu (LC 20 AT VP) HPLC system on Inertsil ODS (150×4.6 mm ID, 3.5 µm particle size) column was used. The instrument is equipped with an auto sampler and UV-Visible detector. A 10 µl rheodyne injector port was used for injecting the samples. Data was analyzed by using Spin chrome software. A Global digital pH meter was used for pH measurements.

Chemicals and solvents

The working standards of Nivolumab and Ipilimumab were provided as gift samples from Chandra Labs, Hyderabad, India. HPLC grade water, orthophosphoric acid and acetonitrile were purchased from E.Merck (India) Ltd., Mumbai, India.

Chromatographic conditions

Acetonitrile and 0.1% orthophosphoric acid (50:50, v/v) was found to be the most suitable mobile phase for ideal chromatographic separation for simultaneous estimation of Nivolumab and Ipilimumab. The solvent mixture was filtered through 0.45 µm membrane filter and sonicated before use. It was pumped through the column at a flow rate of 1.0 ml/min. Injection volume was 10 µl and the column was maintained at a temperature of 27°C. The column was equilibrated by pumping the mobile phase through the column for at least 30 minutes prior to the injection of the drug solution. The detection of the drug was monitored at 225 nm. The run time was set as 8 minutes.

Preparation of buffer

1 ml OPA was measured and make the final volume to 1 lt with HPLC grade water.

Preparation of mobile phase

Mix acetonitrile and buffer in the ratio of 50:50, v/v. Filtered the solution through 0.45 µm membrane filter paper to remove all fine particles and gases. The mobile phase was sonicated for 10 min to remove gases.

Preparation of standard solution

Weigh accurately 100 mg of Nivolumab and 50 mg Ipilimumab working standards are taken into 100 ml volumetric flask add 70 ml of diluent, sonicate for 10 min to dissolve the contents make upto the mark with diluent. Further dilute 5 ml of above solution to 50 ml volumetric flask with diluent.

Preparation of sample solution

Weigh 100 mg of Nivolumab sample and 50 mg of Ipilimumab sample and transferred into a 100 ml volumetric flask. Add 70 ml of diluents sonicate for 15 min to dissolve the contents, diluted volume with diluent. Further diluted 5 ml of above solution to 50 ml volumetric flask make up with diluent.

Procedure

The column was maintained at a temperature of 27°C. The run time was set at 8 minutes. The column was equilibrated by pumping the mobile phase through the column for at least 30 minutes prior to the injection of the drug solutions. Inject 10 µl of the standard and sample solutions six times into the chromatographic system at a flow rate of 1.0 ml/min and the corresponding chromatograms were obtained. From these chromatograms, the average area under the peak of each dilution was computed (Figure 3).
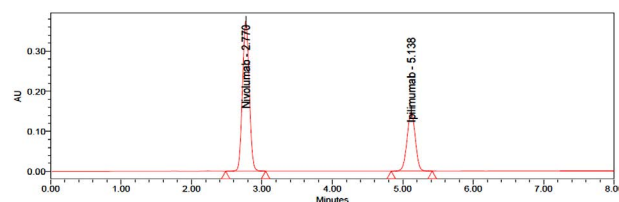
Figure 3: Typical chromatogram of standard for Nivolumab and Ipilimumab

Method validation

Linearity: Several aliquots of standard solutions of Nivolumab and Ipilimumab were taken in six different 10 ml volumetric flasks and diluted up to the mark with diluent such that the final concentrations were in the range of 25-150 µg/ml for Nivolumab and 12.5-75 µg/ml for Ipilimumab. The above solutions were injected into the HPLC system keeping the injection volume constant. The drugs were eluted with UV detector at 225 nm, peak areas was recorded for all the peaks. The linearity curves were constructed by plotting concentration of the drugs against peak areas. The regression equation of this curve was computed. This regression equation was later used to estimate the amount of drugs in tablet dosage forms (Figure 4).

$$y = 25396.61x + 52612.25$$
$$R^2 = 0.9994$$
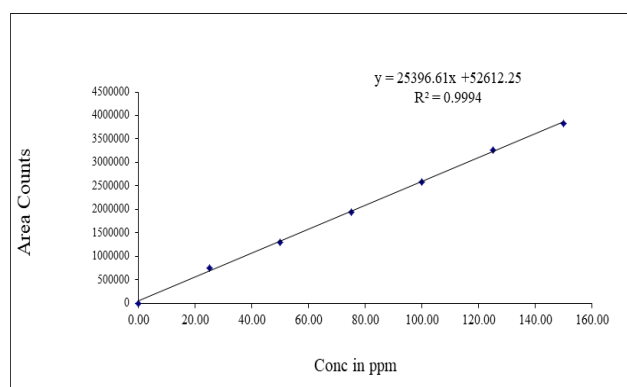
Figure 4: Linearity graph of Nivolumab

Precision: Precision for Nivolumab and Ipilimumab was determined in terms of intra-day precision and inter-day precision. Every sample was injected three times. The measurements for peak areas were expressed in terms of %RSD.

Accuracy

The accuracy of the method was assessed by recovery studies of Nivolumab and Ipilimumab at three concentration levels 50%, 100% and 150%. Fixed amount of pre-ana-

lyzed sample was spiked with known amount of Nivolumab and Ipilimumab. Each level was repeated three times. The %Recovery of Nivolumab and Ipilimumab were calculated.

System suitability

The system suitability parameters like retention time, theoretical plates and tailing factor were evaluated by six replicate analyses of Nivolumab and Ipilimumab and compared with standard values. The acceptance criteria are %RSD of peak areas not more than 2%, theoretical plates numbers (N) at least 2000 per each peak and tailing factors not more than 2.0 for Nivolumab and Ipilimumab.

Limit of detection and limit of quantification

The Limit of Detection (LOD) and Limit of Quantification (LOQ) of the developed method were determined by injecting progressively low concentrations of the standard solutions of Nivolumab and Ipilimumab using the developed HPLC method. LOD and LOQ were estimated from signal to noise ratio. LOD and LOQ were calculated using 3.3 σ/s and 10 σ/s formulae, respectively. Where, σ is the standard deviation of the peak areas and S is the slope of the corresponding calibration curve.

Robustness

The robustness of the method was determined by making small deliberate changes in method like variation of flow rate, mobile phase ratio and temperature.

Solution stability

Changes in the area response of the test material will be monitored throughout the validation. Each solution stability solution shall be injected after 6 hours and 24 hours.

Results and Discussion

The HPLC procedure was optimized with a view to develop an accurate, precise and reproducible method for simultaneous estimation of Nivolumab and Ipilimumab in tablet dosage form using Shimadzu (LC 20 AT VP) HPLC system on Inertsil ODS (150 × 4.6 mm ID, 3.5 μm particle size) column in isocratic mode with mobile phase composition acetonitrile and 0.1% OPA (50:50, v/v) resulted in peak with maximum separation, good shape and resolution. Flow rates between 0.8 to 1.2 ml/min were studied. A flow rate of 1.0 ml/min gave an optimum signal to noise ratio with reasonable separation time, the retention times for Nivolumab and Ipilimumab were found to be 2.770 minutes and 5.118 minutes respectively. Total run time was 8 minutes. The drug components were measured with UV detector at 225 nm. The results of optimized chromatographic conditions were shown in Table 1. The chromatograms were checked for appearance of any extra peaks under optimized conditions, showing no interference from common tablet excipients and impurities. Also, the peak areas were compared with standard and were found to be within limits. As shown in chromatogram, two analytes are eluted by forming symmetrical peaks. The typical chromatogram of Albendazole and Praziquental standard were shown in Fig-

ure 1. Linearity was obtained in the range of 25-150 μg/ml for Nivolumab and 12.5-75 μg/ml for Ipilimumab. The correlation coefficient (r2) was found to be 0.999 for both Nivolumab and Ipilimumab respectively. The regression equation of the linearity plot of concentration of Nivolumab over its peak area was found to be y=25396.61x+52612.25, where x is the concentration of Nivolumab (μg/ml) and y is the corresponding peak area. The regression equation of the linearity plot of concentration of Ipilimumab over its peak area was found to be y=24143.40x+651.61, where x is the concentration of Ipilimumab (μg/ml) and y is the corresponding peak area. The results show that an excellent correlation exists between peak area and concentration of drugs within the concentration range indicated. The linearity results were shown in Tables 2 and 3 and the calibration curves were shown in Figures 3 and 4. The %RSD for system, method and intermediate precision for Nivolumab and Ipilimumab were found to be within the limits (limit %RSD<2.0%) and hence the method is precise. The precision data of Nivolumab and Ipilimumab were furnished in Table 4. The %Recovery of the drugs Nivolumab and Ipilimumab were found to be 99.4 to 100% and 100.5 to 100.7% respectively and the high percentage of recovery of Nivolumab and Ipilimumab indicates that the proposed method is highly accurate. The results of accuracy studies of Nivolumab and Ipilimumab were shown in Table 5. The robustness studies indicated that no considerable effect on the determination of the drugs. Therefore, the test method is robust for the quantification of the drugs. In all deliberately varied conditions, the % RSD for replicate injections of Nivolumab and Ipilimumab were found to be within the acceptable limits. The results of robustness studies of Nivolumab and Ipilimumab were shown in Table 6. The stability studies i.e., changes in the area response of the test material will be monitored throughout the validation. Each solution stability solution shall be injected after 6 hours and 24 hours. The results of the stability studies were shown in Table 7.

Table 1: Optimized chromatographic conditions

S. No.	Parameter	Condition
1	Mobile phase	Acetonitrile and 0.1% OPA 50:50, v/v
2	Column	Inertsil ODS (150×4.6 mm, 3.5 μm)
3	Column temperature	27˚C
4	Wave length	225 nm
5	Injection volume	10 μL
6	Flow rate	1.0 mL/min.
7	Run time	8 min.

Table 2: Linearity studies of Nivolumab

Inj	Std Area
1	2520286
2	2513277
3	2520624
4	2518056
5	2511947
6	2518119

mean	2517052
SD	3624.254
% RSD	0.14

3	1203847
4	1196328
5	1203809
6	1202728
mean	1199129
SD	4929.911
% RSD	0.41

Table 3: Linearity studies of Ipilimumab

Inj	Std Area
1	1195540
2	1192520

Table 4: Precision data of Nivolumab and Ipilimumab

S. No.		Area of Nivolumab	Area of Ipilimumab
System Precision	1	2520286	1195540
	2	2513277	1192520
	3	2520624	1203847
	4	2518056	1196328
	5	2511947	1203809
	6	2518119	1202728
	Mean	2517052	1199129
	SD	3624.25	4929.91
	% RSD	0.144	0.411
Method Precision	1	2501286	1195977
	2	2536871	1206574
	3	2512783	1187354
	4	2562874	1204856
	5	2527136	1215669
	6	2486784	1198983
	Mean	100.2	100.2
	SD	1.069	0.827
	% RSD	1.07	0.83
Intermediate precision	1	2513125	1215379
	2	2515871	1182559
	3	2502154	1206358
	4	2536245	1184652
	5	2481871	1211768
	6	2547652	1203883
	Mean	100	100.2
	SD	0.939	1.143
	% RSD	0.94	1.14

Table 5: Accuracy studies of Nivolumab and Ipilimumab

% Concentration level	Nivolumab			Ipilimumab		
	Conc. added (µg/mL)	Conc. found (µg/mL)	% Recovery	Conc. added (µg/mL)	Conc. found (µg/mL)	% Recovery
50%	50	49.72	99.4	25	25.18	100.7
100%	100	99.87	99.9	50	50.34	100.7
150%	150	149.97	100.0	75	75.36	100.5

Table 6: Robustness data for Nivolumab and Ipilimumab

S. No.	Condition	%RSD of Nivolumab	%RSD of Ipilimumab
1	Flow rate (-)	0.86	1.01
2	Flow rate (+)	0.8	1.12
3	Mobile phase (-)	0.73	0.78

4	Mobile phase (+)	1.15	1.46
5	Temperature (-)	1.5	0.6
6	Temperature (+)	1.43	0.56

Table 7: Solution Stability data

Time in Hrs.	Nivolumab	Ipilimumab	Resolution
INITIAL	2516472	1198154	11.83
6 Hrs	2510374	1195587	11.86
12 Hrs	2502481	1192046	11.91
18 Hrs	2497210	1191533	11.93
24 Hrs	2492023	1185124	11.76

Conclusion

The developed method was validated in terms of accuracy, Linearity and precision. A good linear relationship was observed for Nivolumab and Ipilimumab in the concentration ranges of 25–150 μg/mL for Nivolumab and 12.5-75 μg/mL for Ipilimumab. The correlation coefficient for Nivolumab and Ipilimumab was found to be 0.999. Selectivity experiment showed that there is no interference or overlapping of the peaks either due to diluents with the main peak of Nivolumab and Ipilimumab.

The percentage RSD for precision is <2 which confirms that method is sufficiently precise and the total runtime required for the method is only 8 mins for eluting both Nivolumab and Ipilimumab. The proposed method is simple, fast, accurate, and precise and can be used for routine analysis in quality control of Nivolumab and Ipilimumab.

Source of Support

None

Conflict of Interest

Nil

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