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LC-MS Method Development and Validation for the Estimation of Nivolumab and Relatlimab in Rat Plasma and Its Pharmacokinetic Study

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KEYWORDS

Relatlimab, Rat plasma, Nivolumab, Development, Validation, LC-MS/MS

ABSTRACT:

Melanoma, pulmonary, kidney cell cancer, malignancy of the head and neck, urothelial, malignant colon cancer, esophageal carcinoma of the squamous cells, liver cancer, gastric cancer, and gastrointestinal junction cancer are among the cancers that can be treated with Nivolumab, anti-cancer drug. Therefore, one of the most important fields of contemporary pharmaceutical analysis is the development, validation, and pharmacokinetic investigation of Nivolumab and Relatlimab in rat plasma. The current application aims to show up when pharmacokinetic assessment of Nivolumab and Relatlimab in rat plasma using LC-MS/MS and bioanalytical system validation is carried out. The current application aims to provide evidence up in the occurrence that a pharmacokinetic analysis of Relatlimab and Nivolumab in rat plasma using LC-MS/MS and bioanalytical system validation will be performed. The improved procedure employed a Waters X-bridge phenyl (250mmx4.6mm, 5µm) column with a flow rate of 1 mL/min. The mobile phase in this experiment was a combination of acetonitrile and buffer (1 mL TFA in 1 litre of water). Nivolumab and Relatlimab differentiate themselves by employing Trastuzumab for the internal standard and 10-minute run duration. Relatlimab linearity range covers 10 and 200 % of the rat plasma, while Nivolumab is between 6 and 240 ng/mL and 2-80 ng/mL. The value of R2 for each analyte was 0.999. According to USFDA criteria, our work shows that all requirements, including exactness, recovery, accuracy, and stability, were fulfilled. Pharmacokinetic investigations of Nivolumab and Relatlimab using rat plasma may be examined using this method.

1. Introduction

One anti-cancer drug used to treat several cancer types is Nivolumab, which is marketed under the trade name Opdivo [1]. A humanized IgG4 monoclonal antibody called Nivolumab inhibits PD-1. As a kind of immunotherapy, it functions as an immune checkpoint inhibitor, obstructing a signal that stops T cells from being activated and fighting the malignancy. Peripheral neuropathy, nausea, tiredness, diarrhoea, vomiting, reduced appetite; stomach discomfort, constipation, and musculoskeletal pain are the most frequent adverse effects that occur when chemotherapy is taken in conjunction with it. If the tumor does not have a BRAF mutation, Nivolumab is used as the initial therapy for ineffective or advanced melanoma in conjunction with ipilimumab. If the cancer does have a mutation, Nivolumab is utilized as an additional line of therapy after ipilimumab and a BRAF inhibitor [2]

It is also used to treat small cell lung cancer and malignant epithelial non-small cell lung carcinoma that progresses with or after platinum-based medications [3]. 8.5% of people may have hypothyroidism, whereas 3.7% may have hyperthyroidism. In about 2% of patients using Nivolumab, autoimmune diabetes that resembles type 1 diabetes may develop [4], T cells that were recently stimulated in the human's body immunological response have a protein called PD-1 on their surface. Normally, molecules like PD-L1 or PD-L2, which may attach to PD-1, play a role in controlling the immune system. When they do, they stop the T cell from doing anything, which serves to keep the body from having an overreaction to the immune system [5, 6]. In patients with Hodgkin's lymphoma, there is

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conflicting evidence regarding Nivolumab beneficial effects on overall survival, standard of life, progression-free survival, and full response [7, 8]. The combination of Nivolumab and ipilimumab was tested in patients with stage IV or recurrent non-small cell lung cancer who had not received prior treatment. Nivolumab and Ipilimumab, Nivolumab alone, or conventional chemotherapy was administered in a 1:1:1 ratio to participants with PD-L1 expression levels of 1% or above [9, 10].

A monoclonal antibody called Relatlimab was developed to treat melanoma. It is used to treat melanoma in conjunction with Nivolumab [11]. The deadliest kind of skin cancer, melanoma, arises from melanocytes, which are cells that produce melanin. Rarely, it can happen in the mouth, intestines, or eye, but usually it happens in the skin [12]. The human LAG3 gene encodes the protein known as lymphocyte-activation gene 3, or LAG-3 [13]. A variety of combinations and distinct analysis reports are given, but no publications have been published for the investigation of these medications at the same time [14–22] **Fig.1** shows the chemical structures of Nivolumab.

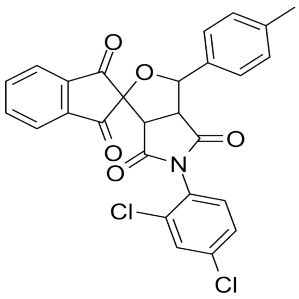

Fig 1 Structural representation of Nivolumab

2. Materials and Method

2.1. Reagents and Chemicals

Nivolumab, Relatlimab, and Trastuzumab specimens from Cipla Pharmaceutical Limited in Vijayawada were supplied as the reference sample. All of the chemicals, including LCMS-grade methanol and acetonitrile, were acquired from Merck Chemical Division in Mumbai. Throughout the investigation, HPLC-grade water from the Milli-Q water purification system was employed.

2.2 Equipment

Chromatography was carried out using a SCIEX QTRAP 5500 mass spectrometer, which is a small device with top-notch ABSCIEX software, and a Waters 2695 HPLC equipped with an extremely high velocity auto sampler, column oven, and degasser.

2.3 Instrumental conditions

2.3.1 LC conditions

Transfer 1ml of TFA into 1 liter water (buffer) was used as mobile phase with a flow rate of 1 mL/min. The present method validation involves gradient procedure of elution with a 10 µ L injection volume.

2.3.2 LC/MS conditions

Nivolumab and Relatlimab were separated in rat plasma using a mass spectrometer fitted with positive ion mode electrospray ionization and multiple reactions monitoring mode. The ideal mass spectrometer parameters are nitrogen as the collision gas, 550°C for the source, 15V and 14V collision energies, 120-250°C drying gas temperature, 5500V ion spray voltage, 10V and 7V entrance and exit potentials, and a dwell time of 1 second

2.4 Preparation of Nivolumab Parent Stock Solution

After weighing 6 mg of the Nivolumab working standard, transfer it to a 10 mL volumetric flask that has been diluted with diluent to volume. 0.8 mL was further diluted with diluent to 10 mL.

2.5 Preparation of Relatlimab Parent Stock Solution

8 mg of the working standard for Relatlimab should be weighed and then put into a 10 ml volumetric flask that has been diluted to volume with diluent. 0.2 ml to 10 ml was further diluted using diluent

2.5.1 Preparation of Nivolumab and Relatlimab Stock Solution

Add 0.1 ml of the parent stock solutions of Nivolumab and Relatlimab to a 10 ml volumetric flask, then dilute with diluent.

2.5.2 Preparation of Internal Standard Stock Solution

Weigh 6 mg of the working standard for Trastuzumab and transfer it to a 10 ml volumetric flask that has been diluted with diluent to volume. 0.4 mL was further diluted with diluent to 10 ml. Fill a 10-milliliter volumetric flask with 0.1 milliliters of the stated above solution, then top it out with diluents.

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2.5.3 Preparation of Standard Solution

500 µl of the standard stock solution was transferred into a 2 ml centrifuged tube. Add 800 µl of acetonitrile, 500 µl of internal standard, and 200 µl of plasma to this. For 20 minutes, centrifuge it. Filter the supernatant liquid and transfer it to an HPLC vial.

2.6 Method validation

2.6.1 Selectivity

Six batches of plasma specimens were analyzed using the LC-MS technique. At LLOQC, the chromatograms of the amounts of spiked plasma specimens were spiked with the blank plasma specimens that had previously been characterized.

2.6.2 Matrix effect

Utilizing the LC-MS/MS technology, six sets of plasma specimens were examined. The quantities of the spiked plasma specimen's chromatograms were compared to the previously described blank plasma specimens at LLOQC.

2.6.3 Dilution integrity

Dilution integrity may be demonstrated by contaminating the matrix with material formation over the ULOQC and merging the selected sample with the blank matrix.

2.6.4 Precision and accuracy

To assess the accuracy and precision of samples from the high-quality control, MQC, the levels of LQC, and LLOQC levels, six duplicates of a single collection were evaluated on the same day. By assessing concentration samples of high-quality control, MQC, LQC, and LLOQC on three distinct batches, the inter-day accuracy and precision were assessed. Precision was stated as a percentage of CV, and accuracy as a percentage of recovery.

2.6.5 Carry over

During sample injection, the chromatographic process maintained the analyte, which was later discovered in blank or unidentified samples.

2.6.6 Recovery

Six duplicates at each quality control concentration are analyzed to determine the removal efficiencies of Relatlimab and Nivolumab. By analyzing the peak outputs of the extracted and non-extracted standards, the recovery percentage was determined.

2.6.7 Stability

The internal norm and area response in the stability sample are contrasted with the area response of a standard solution made from scratch. Six preparations were employed in each of the two stages of amount accumulation low and high for stability testing. The USFDA requires samples to be stable below 15%. Autosamplers were used to preserve benchtop stability samples at 2–8°C for up to 24 hours. Unlike a newly prepared control sample, freeze-thaw stability samples are frozen at -30°C and thawed three times. In order to test for freeze-thaw stability, six different samples with different LQC and HQC levels are used. Wet extract stability samples are kept for a whole day at a temperature below 10°C.

3. 3 Results and Discussion

As the sample is combined within the continuous flow of mobile phase to the electrospray ionization source used at both positive and negative modes at a flow of 10µL/min, the instrument is optimized to provide sensitivity and signal stability (**Fig**.2, 3 and 4). Compared to negative ion mode, Nivolumab and Relatlimab react more in positive ion mode. To achieve the optimal chromatographic condition, several columns, including C18, C8, and CN-propyl, as well as mobile phases made of acetonitrile and water, were tried. The Waters X-bridge phenyl (250mmx4.6mm, 5µm) column with a mobile phase of CH_3CN and 0.1% TFA at a flow rate of 1 mL/min produced the best chromatographic separation.

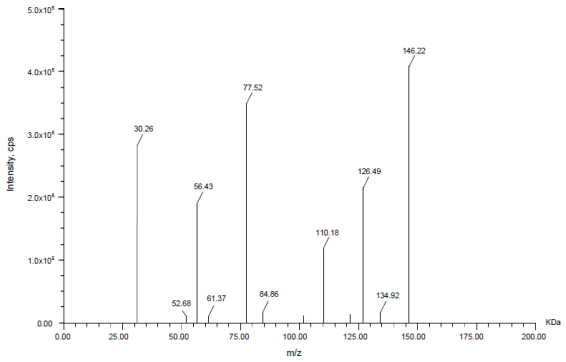

Fig 2: Mass spectra of Nivolumab

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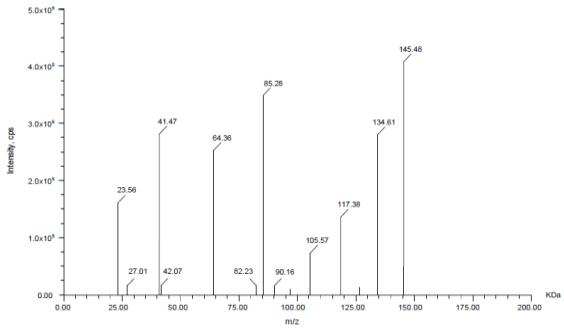

Fig 3: Mass spectra of Relatlimab

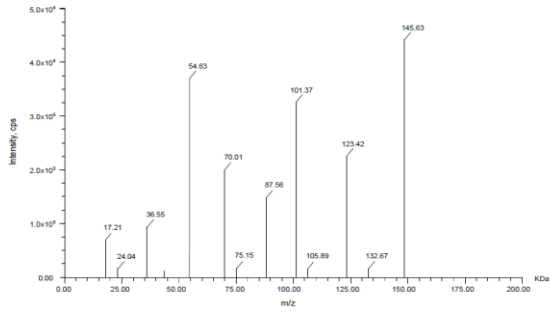

Fig 4: Mass spectra of Trastuzumab (IS)

3.1 Validation

3.1.1 Selectivity and Sensitivity

LOQ samples of Nivolumab and Relatlimab in both spiked and blank plasma. The percentage interference of sample retention time is within acceptable ranges in six distinct types of rat plasma, including lipedemic and hemolyzed plasma, which both include K2EDTA as an anticoagulant for Nivolumab and Relatlimab. Six copies of the extracted samples were created and analyzed at the LLOQC stage in one of the plasma samples that had the least interference during the Nivolumab and Relatlimab retention period. Fig. **5** and **6** show the chromatograms of IS and Blank.

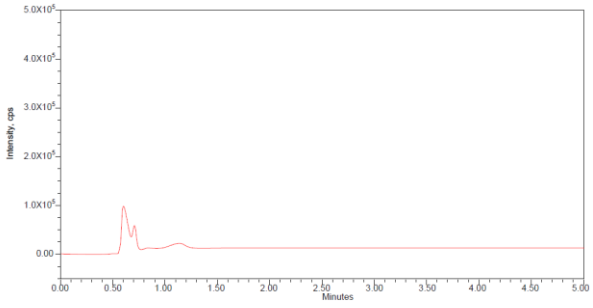

Fig 5 Blank plasma chromatogram

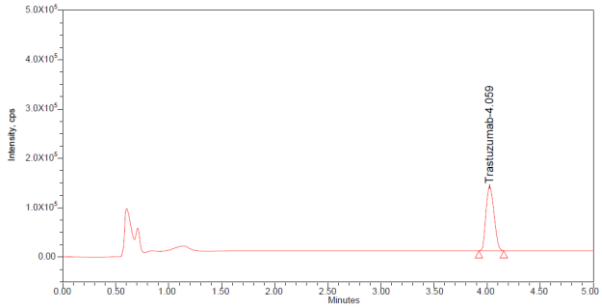

Fig 6 Blank and IS plasma chromatogram

3.1.2 Matrix effect

For Nivolumab and Relatlimab, the ion suppression/enhancement percent CV in the signal was found to be 0.60, 0.69 percent at HQC and 1.88, 1.94 at LQC level. It means that the matrix effect on analyte ionization is within the acceptable range (85.00-115.00 %).

3.1.3 Linearity

The peak response proportions were obviously proportionate with the concentration, as indicated by the calibration curve. The regression coefficients for Relatlimab and Nivolumab were determined to be 0.99969 and 0.99977, respectively. Figure 4 and Tables 1A, B provide specifics of the linearity results for Relatlimab and Nivolumab.

3.1.4 Precision and accuracy

The intra-assay accuracy and precision were evaluated by combining all of the individual reproducible quality control findings from five distinct batch runs on four different days. Nivolumab and Relatlimab had inter-run precision percent CVs of 2.70 and 2.78, respectively, and inter-run accuracy values between 85 and 115 percent. Details of Nivolumab and Relatlimab precision and accuracy are displayed in **Table 2**.

3.1.6 Recovery

Regions for extracted samples with the same concentration levels from a precision and accuracy batch run on the same day were acquired for recovery determination, and LQC, HQC, and MQC concentration levels for Nivolumab and Relatlimab were created. For both ISTD and each QC level, the recovery CV should be less than 15.00 percent. All QC levels should have an overall mean recovery percentage CV of < 20.00%.

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3.1.7 Carry over

One kind of system error that may have an impact on the sample's calculated value is sample carryover. The following procedure was employed to assess sample carryover on an LC-MS/MS equipment that was equipped with Waters Alliance We may thus infer that the accuracy and precision of the suggested approach are unaffected by the use of flow injection analysis to execute a machine blank injection of 10 µL, 0.1 percent TFA, and acetonitrile in a gradient mode on our Zspray triple quadrupole mass detector. Sample carryover was indicated using both % and nL carryover.

3.1.8 Re-injection and reproducibility

In order to confirm the instrument following hardware deactivation owing to any instrument malfunction, re-injection reproducibility was carried out during actual subject sample examination. In the case of an instrument miscarriage during the actual subject test, the batch was reinjected because the alterations were less than 2.0 during the LQC and HQC phases. Samples were produced and reinjected 24 hours later, suggesting that the LQC and HQC levels should vary by less than 2.0 percent. Consequently, the sample can be reinjected after 24 hours in case of an instrument malfunction.

3.1.9 Stability

For solution stability testing, Nivolumab and Relatlimab solutions were made using diluents and kept in a refrigerator between 2 and 8°C. Older stock solutions made during the course of the previous day were contrasted with fresh stock solutions. The percentage fluctuations of Nivolumab and Relatlimab indicate that the stock solutions are stable for up to 24 hours when kept at 2–8°C. Benchtop and auto sampler stabilities were noted at the LQC, MQC, and HQC phases.

Both Nivolumab and Relatlimab were stable for twenty four hours at room temperature in plasma and for twenty four hours at 20°C in an auto sampler. This showed that repeated freezing and thawing had no effect on the stability of plasma samples spiked with Nivolumab and Relatlimab at LQC and HQC levels. Nivolumab and Relatlimab demonstrated long-term stability after 24 hours at a storage temperature of -30°C. Table 3 displays Nivolumab and Relatlimab stability data.

3.1.10 Pharmacokinetic study

Several groups of rats were given oral doses of Nivolumab and Relatlimab while they were fasting. For obtaining concentration-time profiles, obtain samples from the rat's body at intervals of 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, and 14.0 hours after injecting the drug sample (Fig. **7** and **8**). After that, the sample is made in accordance with the test procedure and put into the chromatographic apparatus, where the readings are recorded. The pharmacokinetic variables that had to be examined were Cmax, tmax, and t1/2. Pharmacokinetic information is included in Table 4, and recovery graphs for Relatlimab and Nivolumab are shown in Figures 7 and 8.

Table4: Pharmacokinetic studies of Nivolumab and Relatlimab

Pharmacokinetic parameters	**Nivolumab**	**Relatlimab**
AUC_{0-t}	650 ng-hr/ml	196 ng-hr/ml
C_{max}	112.178 ng/ml	36.270 ng/ml
$AUC_{0-\infty}$	650 ng-hr/ml	196 ng-hr/ml
T_{max}	6 Hrs	6 Hr
$T_{1/2}$	12 Hrs	12 Hrs

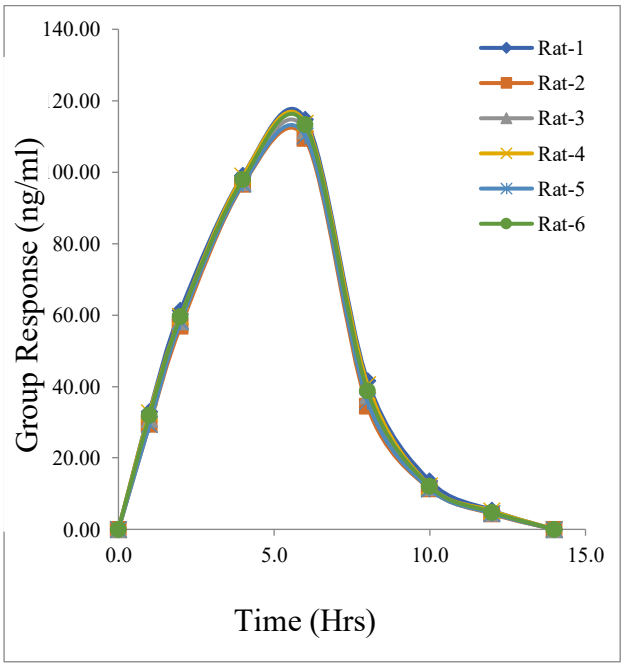

Fig: 7 Recovery plot of Nivolumab

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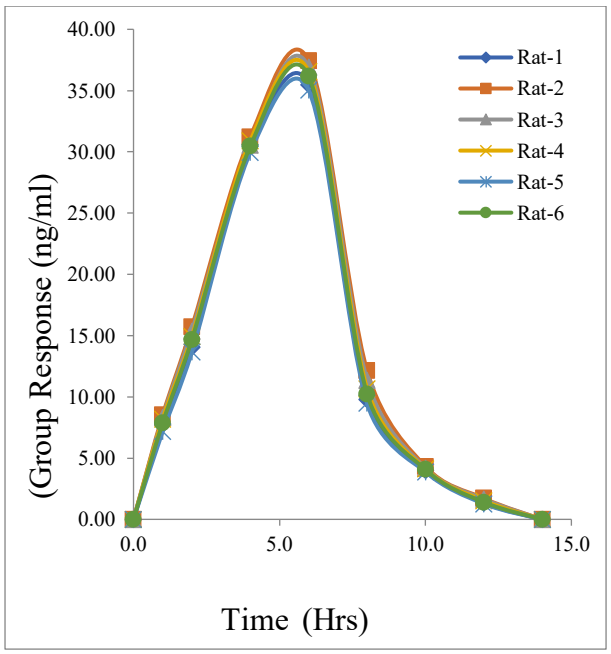

Fig: 8 Recovery plot of Relatlimab

4. Conclusion

The design and testing of a more sensitive LC-MS/MS technique to detect Nivolumab and Relatlimab in rat plasma. The bioanalytical technique now in use is quick, dependable, and repeatable. Furthermore, the suggested approach is straightforward, methodical, and applicable to pharmacokinetic investigations as well as the investigation of the analyte in different body fluids.

Conflicts of interest

The authors did not disclose any conflicts of interest

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