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Research

Analytical method development and validation for the simultaneous estimation of nivolumab and relatlimab in its bulk and pharmaceutical dosage form.

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Abstract

A rapid and precise reverse phase high performance liquid chromatographic method has been developed for the validated of Relatlimab and Nivolumab, in its pure form as well as in tablet dosage form. Chromatography was carried out on X bridge C18 (4.6×150mm) 5µcolumn using a mixture of Methanol: Phosphate Buffer pH3 (60:40v/v) as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 260nm. The retention time of the Relatlimab and Nivolumab was 2.6, 3.8±0.02min respectively. The method produce linear responses in the concentration range of 5-25µg/ml of Relatlimab and 20-100µg/ml of Nivolumab respectively. The method precision for the determination of assay was below 2.0%RSD. The method is useful in the quality control of bulk and pharmaceutical formulations.

Keywords: Relatlimab, Nivolumab, RP-HPLC, Validation, Precision.

INTRODUCTION

Pharmaceutical analysis is traditionally defined as analytical chemistry dealing with drugs both as bulk drug substances and as pharmaceutical products (formulations). Analysis may be defined as the science and art of determining the composition of materials in terms of the elements or compounds contained in them. In fact, analytical chemistry is the science of chemical identification and determination of the composition (atomic, molecular) of substances, materials and their chemical structure.

Chemical compounds and metallic ions are the basic building blocks of all biological structures and processes which are the basis of life. Some of these naturally occurring compounds and ions (endogenous species) are present only in very small amounts in specific regions of the body, while others such as peptides, proteins, carbohydrates, lipids and nucleic acids are found in all parts of the body. The main object of analytical chemistry is to develop scientifically substantiated methods that allow the qualitative and quantitative evaluation of materials

with certain accuracy. Analytical chemistry derives its principles from various branches of science like chemistry, physics, microbiology, nuclear science and electronics. This method provides information about the relative amount of one or more of these components. [1]

Every country has legislation on bulk drugs and their pharmaceutical formulations that sets standards and obligatory quality indices for them. These regulations are presented in separate articles relating to individual drugs and are published in the form of book called "Pharmacopoeia" (e.g. IP, USP, and BP). Quantitative chemical analysis is an important tool to assure that the raw material used and the intermediate products meet the required specifications. Every year number of drugs is introduced into the market. Also quality is important in every product or service, but it is vital in medicines as it involves life.

There is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, report of new toxicities and development of patient resistance and introduction of better drugs by the competitors. Under these conditions standard and analytical procedures for these drugs may not be available in Pharmacopoeias. In instrumental analysis, a physical property of the substance is measured to determine its chemical composition. Pharmaceutical analysis comprises those procedures necessary to determine the identity, strength, quality and purity of substances of therapeutic importance. [2]

Pharmaceutical analysis deals not only with medicaments (drugs and their formulations) but also with their precursors i.e. with the raw material on which degree of purity and quality of medicament depends. The quality of the drug is determined after establishing its authenticity by testing its purity and the quality of pure substance in the drug and its formulations.

Based upon the determination type, there are mainly two types of analytical methods.
They are as follows:

Qualitative analysis: This method is used for the identification of the chemical compounds.

Quantitative analysis: This method is used for the determination of the amount of the sample.

Quality control is a concept which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stages of production. The decision to release or reject a product is based on one or more type of control action. With the growth of pharmaceutical industry during last several years, there has been rapid progress in the field of pharmaceutical analysis involving complex instrumentation. Substance quality and its specifications are based on substance analysis, and that knowledge is later used for quality control (QC) of the substance during full-scale production. Providing simple analytical procedure for complex formulation is a matter of most importance. So, it becomes necessary to develop new analytical methods for such drugs. In brief the reasons for the development of newer methods of drugs analysis are:

1. Manufacturing industries require both qualitative and quantitative analysis to ensure that their raw materials meet certain specifications, and to check the quality of final product. Raw materials are to be checked to ensure that the essential components are present within the predetermined range of composition and there are not any unusual substances present which might upset the manufacturing process or it may appear as a harmful impurity in the final product.
2. In the development of new products which contains mixtures other then the pure material, it is necessary to ascertain composition of mixture which shows the optimum characteristics for which the material has been developed.
3. Geographical surveys require analysis to determine the composition of soil sample and numerous rock samples collected from the field.
4. Most of the industrial processes give rise to pollutants which may cause health related problems. So quantitative analysis of air, water and soil sample should be carried out to determine the level of pollution and to establish the safe limits for pollutants.

INSTRUMENTATION OF HPLC

The basic liquid chromatograph consists of six basic units. The mobile phase supply system, the pump and programmer, the sample valve, the column, the detector and finally a means of presenting and processing the results.

Mobile phase (solvent) reservoirs and solvent degassing

The mobile phase supply system consists of number of reservoirs (200 mL to 1,000 mL in capacity). They are usually constructed of glass or stainless steel materials which are chemically resistant to mobile phase**.**

Mobile phase

Mobile phases in HPLC are usually mixtures of two or more individual solvents. The usual approach is to choose what appears to be the most appropriate column, and then to design a mobile phase that will optimize the retention and selectivity of the system. The two most critical parameters for nonionic mobile phases are strength and selectivity. [8,24]

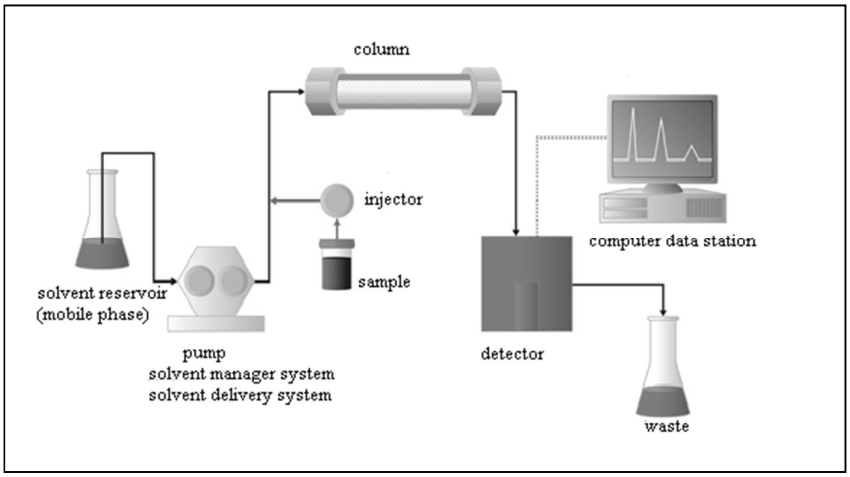

Fig 1: Components of HPLC instrument block diagram[22]

Mobile phase preparation

Mobile phases must be prepared from high purity solvents, including water that must be highly purified. Mobile phases must be filtered through ≤1 µm pore size filters and be degassed before use.

MATERIALS AND METHODS

Relatlimab-Sura labs, Nivolumab-Sura labs, Water and Methanol for HPLC-LICHROSOLV (MERCK), Anhydrous di hydrogen phosphate-Finar chemicals, Phosphate Buffer-Finar chemicals, Citric Acid-Finar chemicals

HPLC METHOD DEVELOPMENT

Mobile Phase Optimization

Initially the mobile phase tried was Water: Methanol and ACN: Methanol with varying proportions. Finally, the mobile phase was optimized to phosphate buffer (pH 3), Methanol in proportion 60:40 v/v respectively.

Optimization of Column

The method was performed with various columns like C18 column ODS column, Zodiac column, and Xterra C18 column. Xbridge C18 (4.6 x 150mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

OPTIMIZED CHROMATOGRAPHIC CONDITIONS

Instrument used : Waters HPLC with auto sampler and PDA detector 996 model.
Column : X bridge C18 (4.6×150mm) 5 µ
Buffer : Phosphate buffer (pH-3)-Dissolve 0.9g of anhydrous dihydrogen phosphate and 1.298g of Citric acid mono hydrate in sufficient water to produce 1000ml. Adjust the pH 3 by using ortho phosphoric acid.
pH : 3
Mobile phase : Methanol: Phosphate Buffer pH3 (60:40v/v)
Flow rate : 1.0 ml per min
Wavelength : 260 nm
Injection volume : 10 µl
Run time : 10 min.

Optimized chromatogram, blank, System suitability parameters are shown in the figure and the results are shown in Table.

PREPARATION OF BUFFER AND MOBILE PHASE

Preparation of Phosphate buffer (pH-3): Dissolve 0.9g of anhydrous di hydrogen phosphate and 1.298 g of Citric acid mono hydrate in sufficient water to produce 1000mL. Adjust the pH 3 by using ortho phosphoric acid.

Preparation of mobile phase: Accurately measured 600 ml (60%) of Methanol and 400 ml of Phosphate buffer (40%) were mixed and degassed in digital ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.
Diluent Preparation: The Mobile phase was used as the diluent.

RESULTS AND DISCUSSIONS

Trial (Optimized Condition)
Mobile phase : Methanol: Phosphate Buffer pH3 (60:40v/v)
Column : X bridge (4.6×150mm, 5 µ)
Flow rate : 1.0 ml/min
Wavelength : 260 nm
Column temp : Ambient
Injection Volume : 10 µl
Run time : 8 min

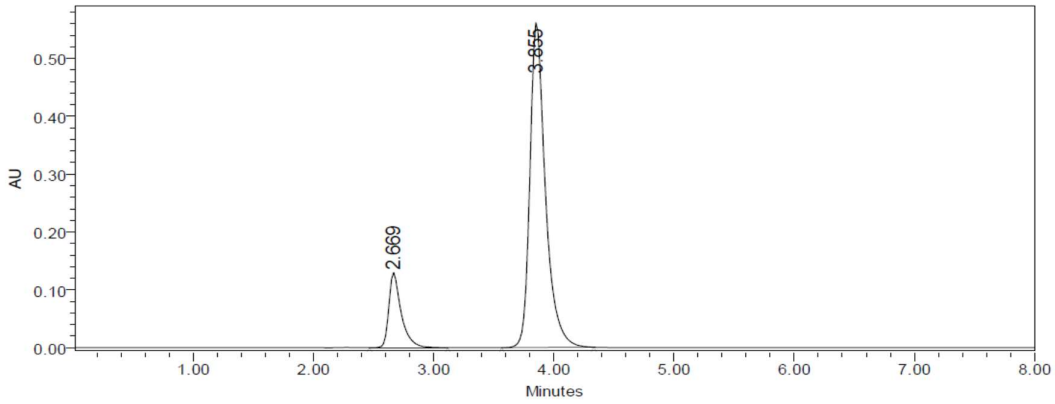

Fig 2: Chromatogram for Trail 7

Table 1: Peak Results for Trail 7

S. No.	Peak name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Relatlimab	2.669	917816	128672		1.5	3551.0
2	Nivolumab	3.855	5040174	562209	1.7	1.4	4675.7

Observation:
This trial shows improper separation sample peaks, baseline and show very less plate count in the chromatogram. So it's required more trials to obtain good peaks. From the above chromatogram it was observed that the Nivolumab and Relatlimab peaks are well separated and they shows proper retention time, resolution, peak tail and plate count. So it's optimized trial.
Retention time of Relatlimab– 2.669min
Retention time of Nivolumab –3.855min

SYSTEM SUITABILITY

Table 2: Results of system suitability parameters for Relatlimab and Nivolumab

S.No.	Name	Retention time(min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	Relatlimab	2.669	918737	128687		1.5	3549.3
2	Nivolumab	3.855	5040174	562209	1.7	1.4	4675.7

- *Resolution between two drugs must be not less than 2.*
- *Theoretical plates must be not less than 2000.*
- *Tailing factor must be not less than 0.9 and not more than 2.*
- *It was found from above data that all the system suitability parameters for developed method were within the limit.*

Assay (Standard)

Table 3: Showing assay standard results

S.No.	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Relatlimab	2.669	918296	128680		1.5	3550	1
2	Nivolumab	3.855	5041296	562209	1.7	1.4	4675	1
3	Relatlimab	2.669	918482	128625		1.5	3548	2
4	Nivolumab	3.855	5040174	562162	1.7	1.4	4592	2
5	Relatlimab	2.654	918215	128721		1.5	3595	3
6	Nivolumab	3.849	5040154	562481	1.7	1.4	4618	3

Assay (Sample)

Table 4: Showing assay sample results

S.No.	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Relatlimab	2.669	918296	128680		1.6	3550.1	1
2	Nivolumab	3.855	50401746	562209	1.7	1.4	4675	1
3	Relatlimab	2.651	919583	128700		1.5	3547.8	2
4	Nivolumab	3.849	15041294	562209	1.7	1.4	4675	2
5	Relatlimab	2.621	918296	128680		1.5	3550.1	3
6	Nivolumab	3.840	5040215	562209	1.7	1.4	4675	3

Table 5: Showing Assay Results

S.No	Name of compound	%purity
1	Relatlimab	98 %
2	Nivolumab	99%

The retention time of Nivolumab and Relatlimab was found to be 2.669min and 3.855mins respectively. The % purity of Relatlimab and Nivolumab in pharmaceutical dosage form was found to be 98% and 99% respectively.

PRECISION

Table 6: Results of method precession for Relatlimab

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Relatlimab	2.669	918296	128680	3550	1.5
2	Relatlimab	2.659	918356	128712	3546	1.5
3	Relatlimab	2.671	918247	128614	3574	1.5
4	Relatlimab	2.669	918636	128647	3564	1.5
5	Relatlimab	2.669	919578	128652	3712	1.5
Mean			918622.6			
Std. Dev			554.9295			
% RSD			0.060409			

Table 7: Results of method precision for Nivolumab

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Nivolumab	3.855	5040174	562209	4675	1.4	1.7
2	Nivolumab	3.842	5046151	562219	4765	1.4	1.7
3	Nivolumab	3.850	5053141	561436	4512	1.4	1.7

4	Nivolumab	3.845	5076521	562148	4155	1.4	1.7
5	Nivolumab	3.855	5063147	571542	4951	1.4	1.7
Mean			5055827				
Std. Dev			14384.71				
% RSD			0.284518				

- *%RSD for sample should be NMT 2.*
- *The %RSD for the standard solution is below 1, which is within the limits hence method is precise.*

INTERMEDIATE PRECISION/RUGGEDNESS

Table 8: Results of Intermediate precision for Relatlimab

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Relatlimab	2.669	918296	128675	3684	1.5
2	Relatlimab	2.529	908296	128457	3564	1.5
3	Relatlimab	2.669	907194	128475	3579	1.5
4	Relatlimab	2.569	909291	128621	3569	1.5
5	Relatlimab	2.569	908296	128632	3546	1.5
6	Relatlimab	2.669	908458	128419	3550	1.5
Mean			909971.8			
Std. Dev			4132.316			
% RSD			0.454115			

Table 9: Results of Intermediate precision for Nivolumab

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Nivolumab	3.845	4940174	562182	4678	1.4	1.7
2	Nivolumab	3.795	4951174	562493	4675	1.4	1.7
3	Nivolumab	3.855	4942175	562198	4624	1.4	1.7
4	Nivolumab	3.840	4840174	563541	4684	1.4	1.7
5	Nivolumab	3.855	4950176	562184	4675	1.4	1.7
6	Nivolumab	3.855	4942312	562487	4621	1.4	1.7
Mean			4927698				
Std. Dev			43117.6				
% RSD			0.875005				

- *%RSD of five different sample solutions should not more than 2*
- *The %RSD obtained is within the limit, hence the method is rugged.*

ACCURACY

Table 10: Accuracy (recovery) data for Relatlimab

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	577153	7.5	7.47	98%	98.8%
100%	918737	15	14.92	99.2%	
150%	1288229	22.5	22.49	99.3%	

- *The percentage recovery was found to be within the limit (98-102%).*

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

Table 11: Accuracy (recovery) data for Nivolumab

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	3120597	30	29.8	98%	99.1%
100%	5040174	60	59.9	99.9%	
150%	7087906	90	89.8	99.6%	

- *The % Recovery for each level should be between 98.0 to 102.0%.*

LINEARITY

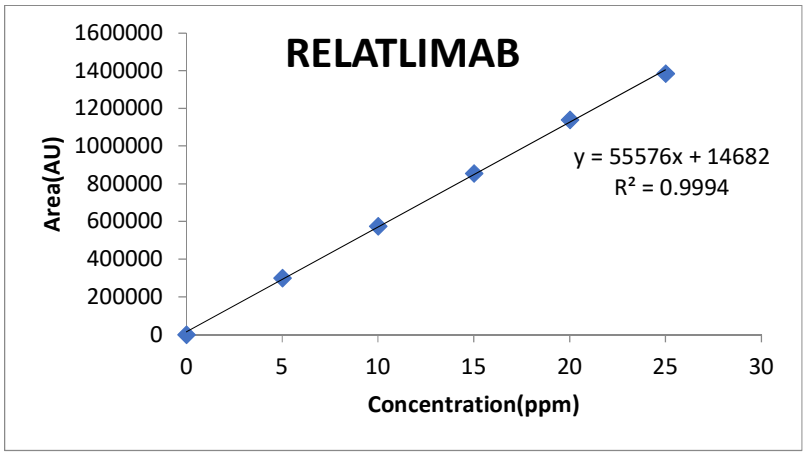

Fig 3: Calibration graph for Relatlimab

Linearity Results: (for Relatlimab)

S.No	Linearity Level	Concentration(ppm)	Area
1	I	5	300010
2	II	10	575361
3	III	15	856266
4	IV	20	1139178
5	V	25	1385477
Correlation Coefficient			0.999

Correlation coefficient should be not less than 0.999

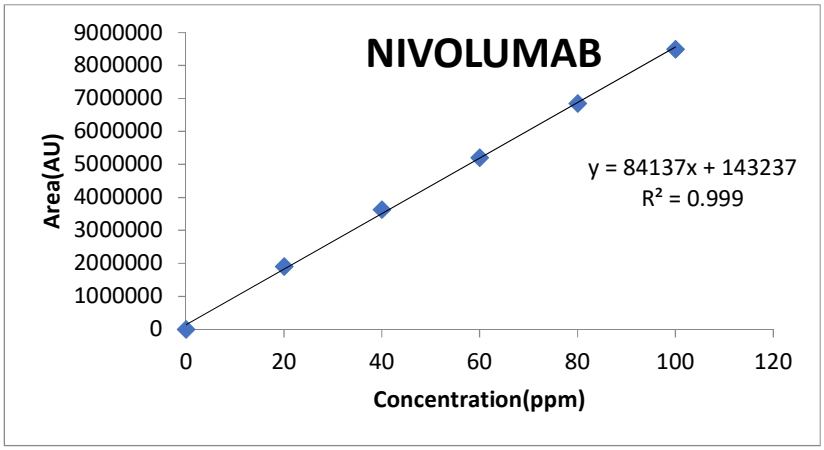

Fig 4: calibration graph for Nivolumab

Linearity Results: (for Nivolumab)

S.No	Linearity Level	Concentration(ppm)	Area
1	I	20	1903922
2	II	40	3637044
3	III	60	5210174
4	IV	80	6856370
5	V	100	8493149
Correlation Coefficient			0.999

Correlation coefficient should be not less than 0.99.

ROBUSTNESS

System suitability results for Relatlimab

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.9	3462	1.5
2	1.0	3578	1.5
3	1.1	3421	1.5

Results for actual flow (1.0 ml/min) have been considered from Assay standard.

System suitability results for Nivolumab

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.9	4675	1.4
2	1.0	4675.6	1.4
3	1.1	4085	1.4

Results for actual flow (1.0ml/min) have been considered from Assay standard.

System suitability

System suitability results for Relatlimab

.No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	4819.3	1.5
2	*Actual	3550.3	1.5
3	10% more	4721.8	1.5

System suitability results for Nivolumab

S.No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	5834.2	1.4
2	*Actual	4675.6	1.4
3	10% more	5235.6	1.4

Results for actual mobile phase have been considered from Assay standard.

CONCLUSION

High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of Relatlimab and Nivolumab was done by RP-HPLC. The Phosphate buffer was p^H 3 and the mobile phase was optimized with consists of Methanol: Phosphate buffer (pH-3) mixed in the ratio of 60:40 % v/ v. An Xbridge column C18 (4.6 x 150mm, 5µm) or equivalent chemically bonded to porous silica particles was used as stationary phase. The solutions were chromatographed at a constant flow rate of 1.0 ml/min. The linearity range of Relatlimab and Nivolumab were found to be from 5-25µg/ml, 20-100µg/ml respectively. Linear regression coefficient was not more than 0.999, 0.999. The values of % RSD are less than 2% indicating accuracy and precision of the method. The percentage recovery varies from 98-99% of Relatlimab and Nivolumab. LOD and LOQ were found to be within limit. The results obtained on the validation parameters met ICH and USP requirements. It inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

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