Scholars Academic Journal of Pharmacy
Abbreviated Key Title: Sch Acad J Pharm
ISSN 2347-9531 (Print) | ISSN 2320-4206 (Online)
Journal homepage: http://saspublishers.com

OPEN ACCESS

Analytical Development

Development and Validation of Analytical Method of Novel Cleaning Validation for Immunomodulating Agent by New RP-HPLC

Mustafa Görkem Bilgin[1*], Bijen İşitmezoğlu[1], Gönül Kayar[1]

[1]Analytical Development Department of R&D Center, Abdi Ibrahim Pharmaceuticals, P.O. Box 34538, Esenyurt, Istanbul, Turkey

DOI: 10.36347/sajp.2023.v12i02.001 | **Received:** 02.11.2022 | **Accepted:** 08.12.2022 | **Published:** 08.02.2023

***Corresponding author:** Mustafa Görkem Bilgin
Analytical Development Department of R&D Center, Abdi Ibrahim Pharmaceuticals, P.O. Box 34538, Esenyurt, Istanbul, Turkey

Abstract **Original Research Article**

The analytical method was developed to evaluate the effectiveness of the procedure to clean the remains of the active pharmaceutical ingredients (APIs) from the equipment surface after manufacture of the final product. For this validation, High Performance Liquid Chromatographic (HPLC) method has been chosen. The HPLC chromatographic separations were achieved using C18 (150×4.6 mm), 5µm, column, employing buffer solution and organic solvent in the ratio of 70:30 (v:v) as mobile phase with the flow rate 1.0 ml/min. The column temperature was maintained at 25 °C and a detector wavelength of 225 nm was employed. Method validation study was conducted on eight separate surfaces. In this paper, stainless steel surface, one of the eight surfaces, was chosen since it is the most widely used surface during the manufacture of the finished product including immunomodulating agent. Validation study was studied between the linearity levels of 2.7% (LOQ% level) and 300%. The method was successfully validated by establishing specificity, linearity, precision (system precision, repeatability, and intermediate precision), accuracy, robustness, solution stability and limit of detection (LOD) & limit of quantification (LOQ) for immunomodulating agent raw material.

Keywords: Immunomodulating agent, cleaning validation, method validation, HPLC.

INTRODUCTION

Drugs known as immunomodulatory agents can either activate or depress the immune system, which may aid the body to fight against cancer, infections, or other disorders. Certain immunomodulating substances, such monoclonal antibodies, cytokines, and vaccinations, have an effect on particular immune system components [1]. Non-specific immunomodulatory drugs have a broad impact on the immune system. They are called an immune system modulator as well. Immunomodulatory drugs are unique family of orally administered anti-cancer medications (IMiD) have significantly changed how patients with hematological malignancies, notably multiple myeloma (MM), are treated [2].

One of the immunomodulatory drugs was synthesized in 1950s in Germany using 2-Aminopentanedioic acid as the starting material. In the early trimester of pregnancy, it was administered as a sedative and hypnotic anti-emetic to alleviate morning sickness. However, these types of drugs were taken off the market in the 1960s due to their side effects when used during pregnancy, including the development of amelia and phocomelia. In late 1990s, the medication was ultimately given U.S. Food and Drug Administration (FDA) approval for the treatment of erythema nodosum leprosum, subject to stringent marketing regulations [3].

Immunobodulating agents have shown critical worse side effects such as neutropenia, thrombocytopenia, anemia, and fatigue on patients who take them reported in the literature [4]. It is very crucial to clean the surfaces on which manufacturing of these substances are performed. If the cleaning processes are brought to completion successfully, immunobodulating agents will not be carried over in the next production of other products on the same surfaces. At this point, it can be concluded that cleaning validation is very essential for two key reasons. One main reason is that it assists in ensuring that there are no carryovers coming from the previous manufacturing of drugs present throughout the production processes. The other reason is that patient's safety is guaranteed for the drug product [5].

Cleaning validation is a formalized procedure that demonstrates how thoroughly, and consistently pharmaceutical production equipment is cleaned [6]. The objective of cleaning validation is to determine if the cleaning system is efficient at removing product deposits, degradants, additives, excipients, or cleaning agents, as well as at preventing potential microbiological contamination that may come from the product that is manufactured in an equipment or a system [7]. The method of cleaning should ensure that undesired contaminants are removed from the spaces and tools utilized for manufacturing. In pharmaceutical companies, cleaning validation is an essential process in this respect. Cleaning validation can be achieved by many analytical techniques. Swab technique is the most commonly used one to make sure that the surface or equipment is clean after a drug production [8]. It has advantages for being used such as accessing the surfaces which are hard to reach easily. For example, sampling with swab is very convenient in isolators, large work surfaces, and corners of equipment. In this article, the swab technique was also preferred to perform the cleaning validation due mainly to its advantages.

HPLC with UV detectors is generally used for the analytical development and validation of cleaning processes as well as assay methods. Cleaning validation methods are sometimes the same as the ones for assays. They are different in that cleaning validation methods include limit of detection and quantification (LOD and LOQ) [9].

In the current study, a novel RP-HPLC technique for a type of immunomodulating agent was developed and efficiently validated by using the swab technique. This study is crucial in order to clean these agents from the production line due to their side effects and thus not interact with other drugs. Up to date, there are no research publications about immunomodulating agents cleaning validation.

MATERIALS AND METHODS

Standards, Reagents, and Samples

The standard (99.8%) that was used for the cleaning validation of the immunomodulating agent was purchased from Hetero Limited Labs. The solvents such as acetonitrile, methanol, and dimethylformamide are obtained from Merck, Darmstadt, Germany. Reagents i.e., phosphoric acid and potassium dihydrogen phosphate used during the validation are purchased from Isolab and Merck, Darmstadt, Germany respectively. All solvents used in validation studies were LC grade.

Experimental

HPLC Chromatographic Parameters

The analytical method of cleaning validation for the immunomodulating agent was developed and validated by using the HPLC from Waters having PDA and UV detector with a specific software, Empower® 3 in which the resulting signals were acquired, and the obtained chromatograms were processed. The column that was used for the cleaning validation was chosen as a reversed phase XDB C18 with dimensions of 150 mm x 4.6 mm and particle size 5 µm (Zorbax® Eclipse). The injection volume was set as 20 µL. The column oven and autosampler temperature were chosen as 25 °C. The mixture of organic solvent and buffer was used as a mobile phase with the flow rate of 1.0 mL/min. Detector wavelength of 225 nm was selected. In this kind of chromatographic study, the retention time of the immunomodulating agent was found out to be approximately 3.4 minutes. For all method validation parameters except for specificity/selectivity parameter, run time was arranged as 6 minutes.

Preparation of Swab Blank

Mix dimethylformamide and deionized water in the ratio of 50 : 50 (v:v). Swab stick is gotten wet using 1 ml of this solution.

Preparation of Diluent

Mix methanol and deionized water in the ratio of 90 : 10 (v:v).

Preparation of Swab Blank Solution

Take 0.5 ml of diluent and transfer to related plate whose surface area is 100 cm^2. Diluent is left to dry on the related surface. The surface is swept with 1 Swab stick that is wet by swab diluent and 1 dry swab stick twice. Take the two swab sticks into a centrifuge tube and add 25 ml of diluent. Vortex for 1 minute. Filter from 0.45 µm Nylon filter and transfer into vial.

Preparation of 2 Swabs + Diluent

Mix 2 swabs, one of which is wetted with 1 ml of swab blank and in 25 ml of diluent.

Preparation of Stock Standard Solution

Weigh 10 mg raw material standard into a 100 ml volumetric flask. Add approximately 50 ml of diluent to dissolve it. Dilute it to volume with diluent (Concentration = 0.1 mg/ml).

Preparation of Standard Solution

Transfer 1.0 ml of stock standard solution into a 50 ml volumetric flask. Dilute it to volume with diluent. Filter it with 0.45 µm Nylon filter, take it into vial (Concentration = 0.002 mg/ml).

Preparation of Swab Surface Sample

The surface is swept with 1 Swab stick that is wet by swab diluent and 1 dry swab stick twice. Take the two swab sticks into a centrifuge tube and add 25 ml of diluent. Vortex for 1 minute. Filter from 0.45 µm Nylon filter and transfer into vial.

Preparation of 100% Spiked Swab Surface Sample

Take 0.5 ml of raw material standard solution and transfer to related plate whose surface area is 100

cm^2. This solution is left to dry on the related surface. The surface is swept with 1 swab stick that is wet by swab diluent and 1 dry swab stick twice. Take the two swab sticks into a centrifuge tube and add 25.0 ml of diluent. Vortex for 1 minute. Filter from 0.45 µm Nylon filter and transfer into vial (Concentration = 0.002 mg/ml).

Method Validation Study

ICH Q2 (R1) guideline is followed when carrying out the cleaning validation of immunomodulating agent. In order to fulfill method validation requirements, specificity and selectivity, linearity and range, detection limit (LOD) and quantification limit (LOQ), precision, accuracy, and robustness parameters were performed. In this study, specificity test was applied to demonstrate that analytical method is capable of measuring the desired substances in a given sample. Linearity of the method was verified by solutions having different known concentrations such as 2.7%, 5.0%, 10.0%, 20.0%, 50.0%, 80.0%, 100.0%, 150.0%, 200.0%, and 300.0% levels which were prepared by diluting the linearity stock solution. The limit of detection (LOD%) and limit of quantification (LOQ%) were calculated from the signal to noise ratio values. Corresponding concentration of LOD was found from signal to noise ratio having the closest value to 3. Corresponding concentration of LOQ was found from signal to noise ratio having the closest value to 10. Accuracy parameter was performed by calculating the recovery per cent of amount of immunomodulating agent obtained from the surface of stainless steels using swab sticks and solution. Robustness parameter was performed by making moderate changes on method parameters such as flow rate, column temperature, and wavelength to show that the obtained results do not vary drastically upon applied changes.

Specificity

Demonstration of capability of analytical method measuring the desired substances was achieved by specificity and selectivity tests. In order to perform this analysis, mobile phase, diluent, swab blanks, swab sticks containing blank, standard, stainless steel sample surface and 100% spiked stainless steel surface sample solutions were prepared. Each prepared solution was injected into the system. There should not be any peak from mobile phase, diluent, swab diluent, swab sticks containing blank, stainless steel sample surface solution at the retention time of main peak (immunomodulating agent) in the chromatogram obtained from standard and sample solutions. Main peak in the chromatogram obtained from standard solution and sample solution should meet purity angle < purity threshold criteria.

Linearity

The capacity of an analytical method to generate results that are directly proportional to the concentration of the analyte in the sample can be used to describe the linearity of an analytical method. Linearity solutions at 10 different concentrations (LOQ%, 5%, 10%, 20%, 50%, 80%, 100%, 150%, 200%, 300%) were prepared to verify linearity. By injecting a series of diluted stock solution, 10 separate concentrations between LOQ% level and 300% of the intended working range were required to evaluate linearity. The linearity graph was plotted by using the average peak area and the concentrations of solutions having different concentrations. In addition, correlation coefficient, slope, and intercept values were reported. The linearity correlation coefficient was evaluated to meet the requirement of this parameter. It should not be less than 0.99.

The Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD is defined as the lowest concentration of a substance in a given sample that can be detected under the test circumstances, but not used for quantifications. The lowest concentration of the substance that can be quantified with an acceptable accuracy and precision is called as LOQ. In this study, LOD and LOQ concentrations are determined according to signal to noise (s/n) method. The general signal to noise that is accepted to calculate the LOD concentration is 3:1 while necessary signal to noise 10:1 is looked for determining LOQ concentration. The peaks obtained from LOD level should be detectable. Signal to noise ratio of main peak obtained from LOD solution injections should be at least 3. Signal to noise ratio of main peak obtained from LOQ solution injections should be at least 10. The relative standard deviation (RSD%) of areas obtained from main peak in the chromatograms obtained from injection performed after LOQ% level determination should be less than 10.0%.

Precision – System Precision

A number of consecutive injections of standard solution prepared at 100% concentration is injected to HPLC system to calculate the mean of main peak areas, relative standard deviation (RSD%), symmetry factor, and theoretical plate counts. The relative standard deviation (RSD%) of main peak areas in the chromatograms obtained from six consecutive injections of standard solution should not be more than 2.0%.

Precision – Repeatability

The capacity of a method to generate consistent results across several preparations of the same sample is known as repeatability. In this study, six stainless steel surface sample solutions at 100% level were prepared to see the repeatability between the results. The relative standard deviation (RSD%) between six sample results should be less than 10.0%.

Precision – Intermediate Precision

The intermediate precision parameter in a method validation study is performed to carry out the precision study by a different analyst, using a different equipment and a column on a different day. The same sample and method parameters were applied to conduct the analysis. Six stainless steel surface sample solutions at 100% level were prepared to see the repeatability between the results.

The relative standard deviation (RSD%) between six sample results should be less than 10.0%. The relative standard deviation (RSD%) of twelve results obtained from repeatability and intermediate precision for each surface should be less than 10.0%.

Accuracy

The accuracy of an analytical method is the degree of closeness between the 'true' value of analytes in the sample and the value determined by the method. The accuracy of the method is shown on stainless steel surface on which raw material is added. This study is performed by preparing three samples of LOQ%, 100% and 300% levels. The recovery % should be between 50.0% and 150.0% for all levels. RSD% should not be more than 10.0% for 9 sample solutions.

Robustness

Robustness is the capacity of a method to remain unaffected by deliberate variations in method parameters such as change in flow rate, wavelength, and column oven temperatures. Sample results as well as retention time, symmetry factor, and theoretical plate count of the main peak which are the one of the main system suitability parameters should not be affected adversely by the changes done on the method parameters. Robustness parameter comprises also solution and mobile phase stability studies.

Robustness - Solution Stability

The stability of the solution refers to the stability of the standard and sample solution and examined according to the method that is used for validation study. Solutions whose stabilities are tested should be stored at room temperature to evaluate the duration in which they are stable. Stability of standard and sample solutions is evaluated as minimum 48 hours. Similarity % of standard and sample solutions should be in the range of 95.0% - 105.0%.

Robustness - Mobile Phase Stability

Likewise, solution stability, stability of mobile phase is evaluated at various time intervals at room temperature. Evaluation of mobile phase should be terminated at the time when the system suitability parameters cannot meet system suitability requirements. Similarity % of standard and sample solutions should be in the range of 95.0% - 105.0%.

RESULTS AND DISCUSSIONS

Method Validation

Specificity

There was no interference of mobile phase, blank, swab blanks, swab sticks containing blank with the main peak. For immunomodulating peak in standard and 100% spiked swab surface sample chromatograms, purity angle < purity threshold criteria were successfully achieved. Therefore, the method was found out to be specific. The specificity results were given in Table-1 and corresponding chromatograms were shown in Figure-1 and Figure-2.

Linearity

Procedure

The concentration of 100% level solution is 0.002 mg/ml. The dilution schemes of other levels of solutions that were used is shown as follows:

Preparation of stock linearity solution was prepared as weighing about 10 mg standard into a 100 ml volumetric flask. Add approximately 50 ml of diluent to dissolve it. Dilute it to volume with diluent (Concentration = 0.1 mg/ml).

Slope, y-intercept, and correlation coefficient determination (R) values were determined and shown in Table-2. The average area values of twicely injected linearity solutions were given in Table-3 and an example chromatogram that belongs to 100% level solution is given in Figure-3. Linearity graph for the main peak is provided in Figure-4. As can be seen from the R^2 value that the linearity of the method is proven.

Table-1: Peak purity of components in Standard and Spiked Sample Solution

Name of the Solutions	Retention Time (min)	Area	Purity Angle	Purity Threshold	Purity Criteria
Standard	3.464	203046	1.775	8.848	Pass
100% Spiked Swab Surface Sample	3.465	196871	2.139	12.254	Pass

Table-2: Data Analysis Summary of Linearity Parameter

Summary Output	
Regression Statistics	
Multiple R	1.00
R Square	1.00
Adjusted R Square	1.00
Standard Error	2703.34
Observation	10
ANOVA	

	df	**SS**	**MS**	**F**	**Significance F**
Regression	1	$3.59x10^{11}$	$3.59x10^{11}$	$4.91x10^{4}$	$1.93x10^{-16}$
Residual	8	$5.85x10^{7}$	$7.31x10^{6}$	-	
Total	**9**	**$3.59x10^{11}$**	**-**		

	Coefficient	**Standard Error**	**t Stat**	**P-Value**
Intercept	2725.70	1196.84	2.28	0.05
X Variable 1	100499729.74	453656.46	221.53	0.00

	Lower 95%	**Upper 95%**
Intercept	-34.22	5485.62
X Variable 1	99453596.06	101545863.42

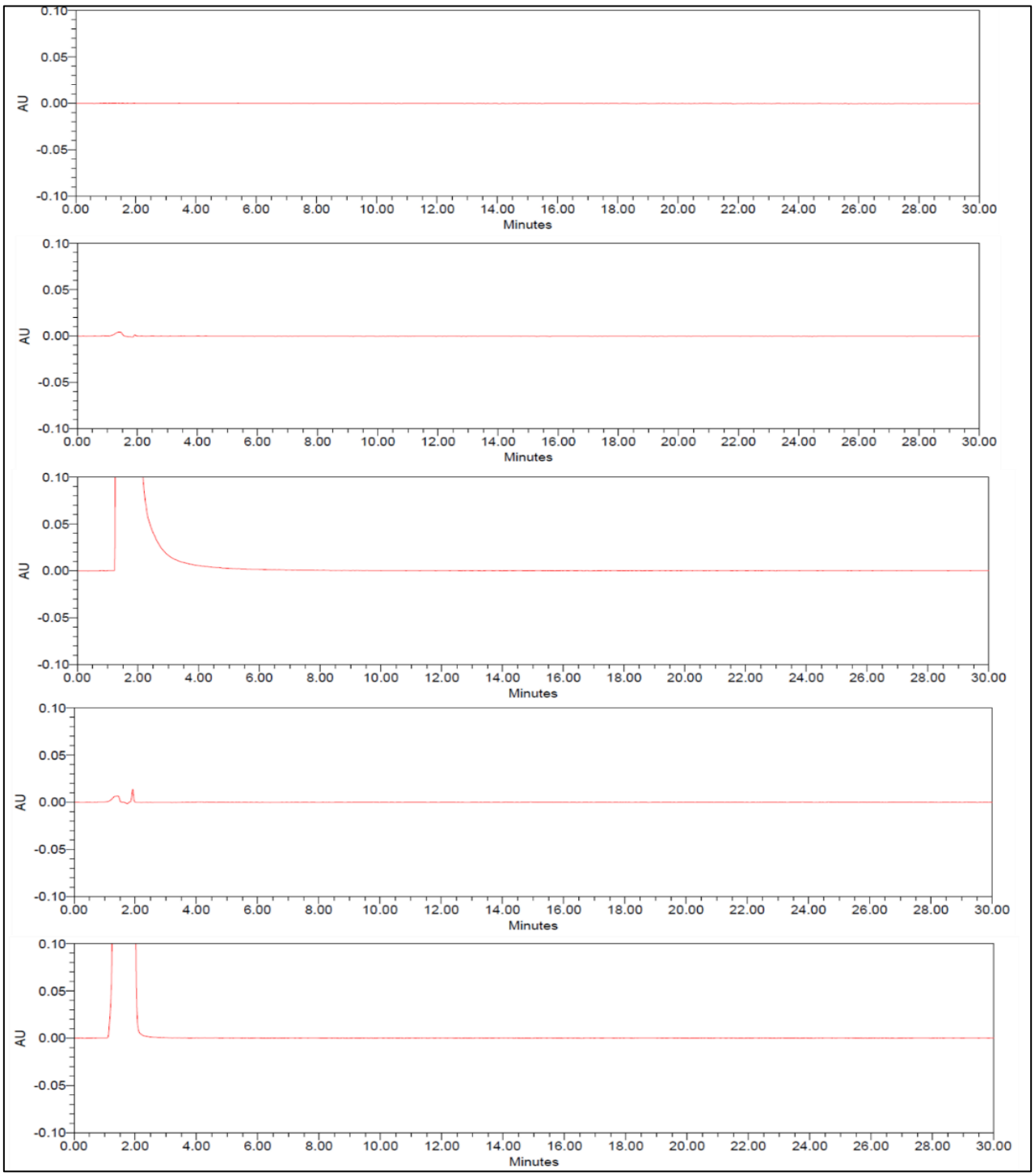

Figure-1: Selectivity Chromatograms: mobile phase, blank, swab blanks, blank containing swab sticks, and stainless-steel sample surface, respectively

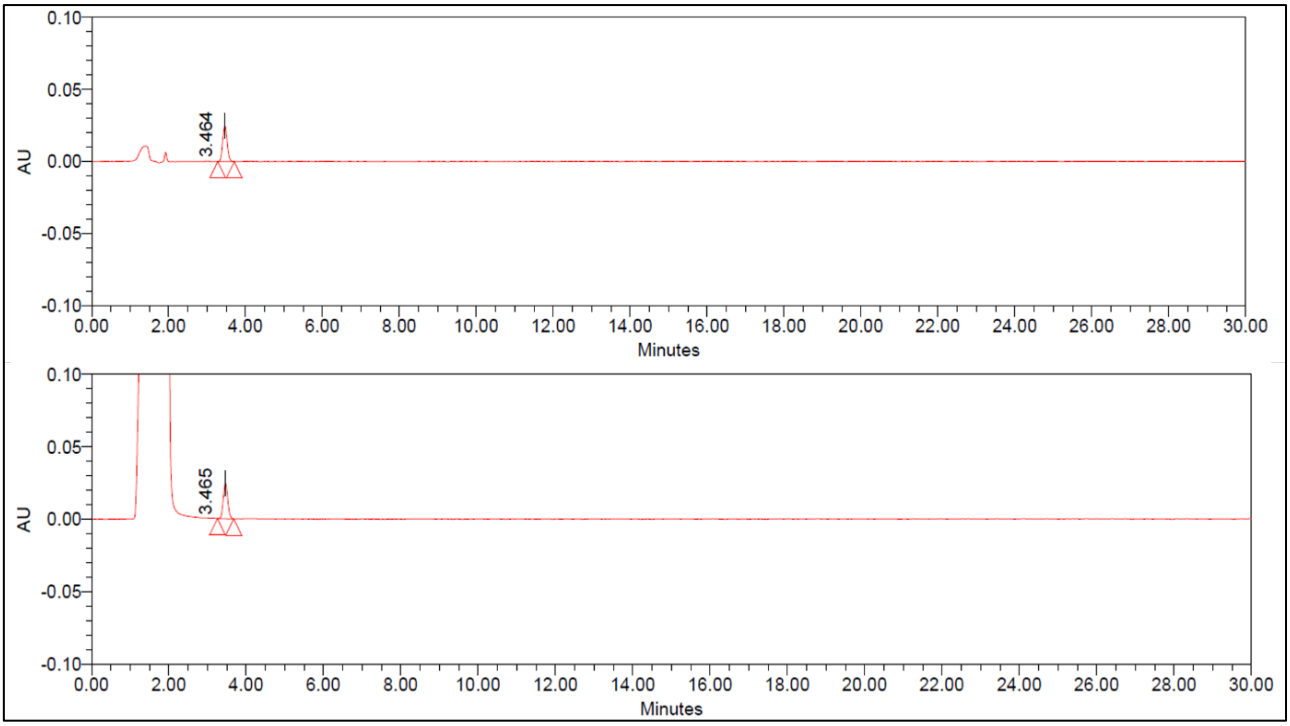

Figure-2: Selectivity Chromatograms: standard and stainless-steel sample surface at 100% level, respectively

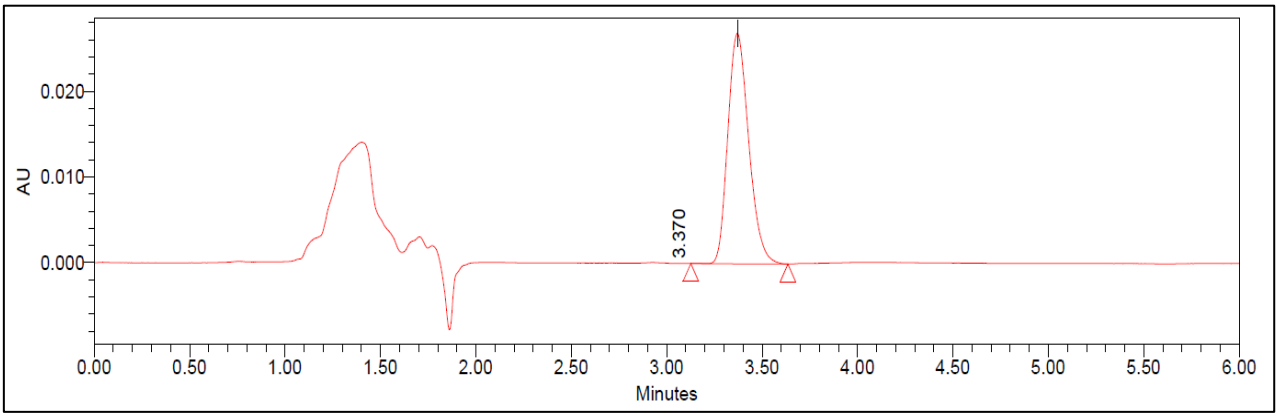

Figure-3: Chromatogram of 100% Level Linearity Solution

Table-3: Results of Linearity Parameter

Level (%)	Actual Concentration (mg/ml)	Mean Peak Area
2.7% (LOQ%)	0.000053920742	5055
5.0%	0.000105984000	12190
10.0%	0.000201196800	22493
20.0%	0.000402393600	42820
50.0%	0.001005984000	107402
80.0%	0.001609574400	167263
100.0%	0.002011968000	204777
150.0%	0.003017952000	308306
200.0%	0.004023936000	402440
300.0%	0.006035904000	610138
Correlation Coefficient		1.000
Slope		100527682.0239
Intercept		2625.6985

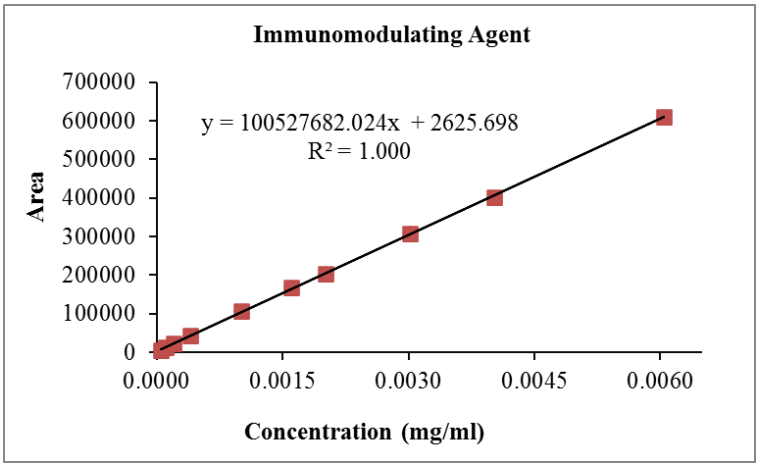

Figure-4: **Linearity Graph of Immunomodulating Agent**

The Limit of Detection (LOD) and Limit of Quantitation (LOQ)

To determine the lowest acceptable value of the analyte where the main peak is detected (LOD) and lowest calculable amount (LOQ) with an acceptable accuracy and precision, this study was performed. The relative standard deviation (RSD%) of the main peak at the LOQ level was found to be less than 10.0%. In addition, s/n values that correspond to LOD and LOQ levels were obtained as more than 3 and 10, respectively. The obtained data for the mean peak was given in Table-4 and Table-5 shown below.

Table-4: **Limit of Detection (LOD) Parameter Results**

Solution Name	LOD (%)	Concentration (mg/ml)	Peak Area	s/n Values
LOD-1	0.8	0.000016	1468	3.2
LOD-2			1452	3.2
Mean			**1460**	**3.2**

Table-5: Limit of Quantification (LOQ) Parameter Results

Solution Name	LOQ (%)	Concentration (mg/ml)	Peak Area	s/n Values
LOQ-1	2.7	0.000054	5110	12.9
LOQ-2			4999	12.8
LOQ-3			4966	12.8
LOQ-4			5119	12.4
LOQ-5			5979	10.7
LOQ-6			4852	12.7
Mean			**5171**	**12.4**
RSD %			**7.9**	

Precision - System Precision

The precision was determined by injecting a series of standards. In this parameter, symmetry factor, theoretical plate count, retention time, area, and confidence interval at 95% of the main peak were reported. The relative standard deviation (RSD %) value of the main peak areas in the chromatograms obtained from six consecutive injections of standard solution was found to be less than 2.0 %. The corresponding data is given in Table-6 shown below:

Table-6: Precision - System Precision Parameter Results

Injection No	Symmetry Factor	Theoretical Plate Count	Retention Time (min.)	Area
1	1.3	5232	3.434	201017
2	1.3	5212	3.435	201149
3	1.3	5258	3.435	201168
4	1.3	5257	3.436	201160
5	1.3	5221	3.433	201297
6	1.3	5262	3.439	201496
Mean	**1.3**	**5240**	**3.435**	**201214**
SD			**0.05**	**164.03**
RSD%			**0.060**	**0.1**
Confidence Interval (95%)			**3.435 ± 0.002**	**201214 ± 131**

Precision - Repeatability

For repeatability parameter, six separate spiked stainless steel surface sample solutions at 100% level having the concentration 0.002 mg/ml. were prepared. The RSD% value which was found less than 10.0% between the analysis results of 6 samples demonstrates that repeatability of the method is proven. The RSD and mean recovery % obtained from six spiked sample solutions are provided in Table-7 below.

Table-7: Precision - Repeatability Parameter Results

Sample No	**Mean Recovery Results (%)**
Sample Solution Spiked at 100% Level -1	96.0
Sample Solution Spiked at 100% Level -2	96.5
Sample Solution Spiked at 100% Level -3	96.4
Sample Solution Spiked at 100% Level -4	96.5
Sample Solution Spiked at 100% Level -5	96.4
Sample Solution Spiked at 100% Level -6	96.4
Mean	**96.4**
SD	**0.19**
RSD %	**0.19**
Confidence Interval (95%)	**96.4 ± 0.2**

Precision - Intermediate Precision

For intermediate precision parameter, six separate spiked stainless steel surface sample solutions at 100% level having the concentration 0.002 mg/ml. were prepared and analyzed on a different day, by different analyst, using a different equipment and column. The relative standard deviation (RSD%) value between the analysis result of 6 samples was found as less than 10.0%. Moreover, RSD% value between results of 12 samples obtained from repeatability and intermediate precision tests was calculated as less than 10.0%. The corresponding results for intermediate precision as well as comparison to results of repeatability parameter are given in Table-8 and Table-9.

Table-8: **Precision - Intermediate Precision Parameter Results**

Sample Name	**Mean Recovery Results (%)**
Sample Solution Spiked at 100% Level -1	91.5
Sample Solution Spiked at 100% Level -2	92.1
Sample Solution Spiked at 100% Level -3	92.1
Sample Solution Spiked at 100% Level -4	91.9
Sample Solution Spiked at 100% Level -5	90.7
Sample Solution Spiked at 100% Level -6	92.0
Mean	**91.7**
SD	**0.55**
RSD %	**0.59**
Confidence Interval (95%)	**92.0 ± 0.4**

Table-9: **Repeatability and Intermediate Precision Parameter Comparison Table**

Sample Name	**Mean Recovery Results (%)**	
	Repeatability	**Intermediate Precision**
	Day-1 Analyst-1 Column-1 Instrument-1	**Day-2 Analyst-2 Column-2 Instrument-2**
Sample Solution Spiked at 100% Level -1	96.0	91.5
Sample Solution Spiked at 100% Level -2	96.5	92.1
Sample Solution Spiked at 100% Level -3	96.4	92.1
Sample Solution Spiked at 100% Level -4	96.5	91.9
Sample Solution Spiked at 100% Level -5	96.4	90.7
Sample Solution Spiked at 100% Level -6	96.4	92.0
Mean	**94.0**	
SD	**2.46**	
RSD %	**2.62**	
Confidence Interval (95%)	**94.0 ± 1.4**	

Accuracy

The accuracy of the method was shown on the stainless-steel plate by adding immunomodulating raw material for LOQ%, 100% and 300%. 3 samples for each level as total 9 samples were prepared. RSD % between recovery results obtained from LOQ%, 100%, and 300% levels were found to be less than 10.0%. The corresponding recovery % results of 9 samples were given in Table-10.

Table-10: **Accuracy Parameter Results**

Sample Name	**Mean Recovery Results (%)**
Sample Solution Spiked at LOQ% Level -1	90.7
Sample Solution Spiked at LOQ% Level -2	91.0
Sample Solution Spiked at LOQ% Level -3	90.8
Sample Solution Spiked at 100% Level -1	96.0
Sample Solution Spiked at 100% Level -2	96.5
Sample Solution Spiked at 100% Level -3	96.4
Sample Solution Spiked at 300% Level -1	89.6
Sample Solution Spiked at 300% Level -2	89.4
Sample Solution Spiked at 300% Level -3	89.4
Mean	**92.2**
SD	**3.14**
RSD %	**3.40**
Confidence Interval (95%)	**92.2 ± 2.1**

Robustness

The robustness of the method was tested, and analysis was performed by applying for deliberate changes on analysis conditions. According to the obtained results, the method was found out as robust against these changes such as flow rate, wavelength, and column temperatures. % Recovery results obtained from repeatability and robustness analysis and peak performance parameters were observed as similar. Since there were no significant changes in the % results, it can be concluded that the method is robust to these deliberate changes. The system precision and recovery % results of robustness parameter are supplied in Table-11 and Table-12.

Table-11: **Robustness Parameter System Suitability Results**

Name of Analysis	**Symmetry Factor**	**Theoretical Plate Count**	**Retention Time (min.)**	**Area**
Repeatability	1.3	5240	3.435	201214
Flow Rate: 0.8 ml/min.	1.3	5338	4.255	250645
Flow Rate: 1.2 ml/min.	1.3	5079	2.843	166386
Wavelength: 223 nm	1.2	4123	3.465	198286
Wavelength: 227 nm	1.2	4128	3.457	199581
Column Temperature: 23°C	1.3	5241	3.480	203910
Column Temperature: 27°C	1.3	5172	3.339	205768

Table-12: Robustness Parameter Recovery Results

Name of Analysis	**Recovery (%)**
Repeatability	96.4
Flow Rate: 0.8 ml/min.	90.9
Flow Rate: 1.2 ml/min.	90.2
Wavelength: 223 nm	92.5
Wavelength: 227 nm	92.8
Column Temperature: 23°C	91.9
Column Temperature: 27°C	90.4

Robustness - Solution Stability

For solution stability parameter, standard and sample solutions were analyzed by keeping the conditions constant at room temperature (25°C) for minimum 48 hours. It has been concluded that standard solution is stable up to 36 hours and sample solution is stable up to 24 hours at room temperature. The corresponding data for system suitability parameters of standard solution and area of sample solution are given in Table-13 and Table-14.

Table-13: **Solution Stability Parameter System Suitability Results**

Hours	Symmetry Factor	Theoretical Plate Count	Retention Time (min.)	Retention Time Similarity (%)	Area	Area Similarity (%)
0th Hour (Initial)	1.3	4979	3.389	-	201868	-
6th Hour	1.3	4857	3.392	100.1	203626	100.9
12th Hour	1.3	4900	3.392	100.1	200153	99.2
18th Hour	1.3	4965	3.394	100.1	196342	97.3
24th Hour	1.3	4841	3.395	100.2	198215	98.2
30th Hour	1.3	4816	3.394	100.1	194890	96.5
36th Hour	1.2	4921	3.397	100.2	192303	95.3
48th Hour	1.3	4910	3.399	100.3	191036	94.6

Table-14: **Solution Stability Parameter Area and Retention Time Similarity Results**

Hours	Area	Area Similarity (%)	Retention Time (min.)	Retention Time Similarity (%)
0th Hour(Initial)	174288	-	3.391	-
6th Hour	171442	98.4	3.393	100.1
12th Hour	171558	98.4	3.393	100.1
18th Hour	168056	96.4	3.395	100.1
24th Hour	166268	95.4	3.395	100.1
30th Hour	164033	94.1	3.397	100.2
36th Hour	162472	93.2	3.397	100.2
48th Hour	157276	90.2	3.400	100.3

Robustness - Mobile Phase Stability

For mobile phase stability parameter, standard and sample solutions were prepared. Standard and sample solution injections were performed with same mobile phase by keeping analysis conditions constant. In order to evaluate the stability of mobile phase, the necessary injections were given on the 7th day and 10th day. It has been concluded that mobile phase is stable up to 10 days at room temperature. No formation of foreign particles was observed in both standard and sample solution at the end of 10 days. The obtained results for standard and sample solutions are shown in Table-15 and Table-16.

Table-15: Mobile Phase Stability Parameter System Suitability Results for Standard Solution

Day	Symmetry Factor	Theoretical Plate Count	Retention Time (min.)	Retention Time Similarity (%)	Area	Area Similarity (%)
Initial Day	1.2	4993	3.389	-	198263	-
7th Day	1.3	4925	3.461	102.1	202249	99.8
10th Day	1.3	4948	3.417	100.8	193756	98.0

Table-16: **Mobile Phase Stability Parameter System Suitability Results for Sample Solution**

Day	Retention Time (min.)	Retention Time Similarity (%)	Area	Area Similarity (%)
Initial Day	3.392	-	174043	-
7th Day	3.462	102.0	175247	100.6
10th Day	3.417	100.7	178085	100.3

CONCLUSION

In this article, we provided a straightforward, accurate, and verified gradient RP-HPLC method for assessing the cleaning validation for a particular immunomodulating agent. Following ICH recommendations, satisfactory validation parameters for linearity, precision, accuracy, LOD, and LOQ were achieved [10]. Therefore, pharma production laboratories and routine monitoring might all benefit from the suggested analytical proposed method.

REFERENCES

1. Bascones-Martinez, A., Mattila, R., Gomez-Font, R., & Meurman, J. (2014). Immunomodulatory drugs: Oral and systemic adverse effects. *Medicina Oral Patología Oral Y Cirugia Bucal*, e24–e31.
2. Chanan-Khan, A. A., Swaika, A., Paulus, A., Kumar, S. K., Mikhael, J. R., Rajkumar, S. V., Dispenzieri, A., & Lacy, M. Q. (2013). Pomalidomide: the new immunomodulatory agent for the treatment of multiple myeloma. *Blood Cancer Journal*, 3(9), e143–e143.
3. Pan, B., & Lentzsch, S. (2012). The application and biology of immunomodulatory drugs (IMiDs) in cancer. *Pharmacology &Amp; Therapeutics*, 136(1), 56–68.
4. Lacy, M. Q., Hayman, S. R., Gertz, M. A., Short, K. D., Dispenzieri, A., Kumar, S., Greipp, P. R., Lust, J. A., Russell, S. J., Dingli, D., Zeldenrust, S.,

Fonseca, R., Bergsagel, P. L., Roy, V., Mikhael, J. R., Stewart, A. K., Laumann, K., Allred, J. B., Mandrekar, S. J., & Buadi, F. (2010). Pomalidomide (CC4047) plus low dose dexamethasone (Pom/dex) is active and well tolerated in lenalidomide refractory multiple myeloma (MM). *Leukemia*, 24(11), 1934–1939.

5. Singh, K., Tamta, B., & Mukopadayay, S. (2022). Cleaning validation process in pharmaceutical industry. *International Journal of Health Sciences*, 13557–13573.
6. Lakshmana, S. P., & Anna University-Tiruchirappalli. (2010b). Cleaning Validation and its importance in Pharmaceutical Industry. *Pharma Times*, 42(07), 21–25.
7. Sharma, S., Goyal, S., & Chauhan, K. (2018). A Review on Analytical Method Development And Validation. *International Journal of Applied Pharmaceutics*, 10(6), 8.
8. Chikanbanjar, N., Semwal, N., & Jyakhwa, U. (2020). A Review Article on Analytical Method Validation. *Journal of Pharma Innovation*, 1(1), 48–58.
9. Brian, F., & Nicholas, H. S. (2007). HPLC with Charged Aerosol Detection for Pharmaceutical Cleaning Validation. *LCGC North America*, 25(9), 960–968.
10. International Conference on Harmonisation. ICH harmonised tripartite guideline for statistical principles for clinical trials. Brookwood Medical Publ. 1998.