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Dual Platform Analytical Strategy for the Simultaneous Quantification of Methotrexate and Leflunomide in Synthetic Mixture using UV Spectrophotometry and RP-HPLC method

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ABSTRACT

Objective: The present article describes efficient, sensitive, and validated UV spectrophotometric and RP-HPLC approach has been developed for the simultaneous determination of Methotrexate and Leflunomide in a synthetic mixture. **Method and Results:** The first-order derivative UV spectrophotometric method was applied using 267.09 nm (zero-crossing point for Methotrexate) for the estimation of Leflunomide, and 232.74 nm (zero-crossing point for Leflunomide) for Methotrexate. The method exhibited linearity over the concentration ranges of 5–25 µg/mL for Methotrexate and 4–20 µg/mL for Leflunomide, with correlation coefficients (R^2) of 0.999 and 0.997, respectively. The mean percentage recoveries were 99.48–99.68% for Methotrexate and 99.69–99.75% for Leflunomide, demonstrating accuracy and reliability. For RP-HPLC analysis, separation was achieved on a Kromstar Vertex C_{18} column (250 × 4.6 mm, 5 µm) in isocratic mode, using Methanol: Water (90:10 %v/v) adjusted to pH 2.5 with orthophosphoric acid as the mobile phase. The flow rate was 1.0 mL/min, and effluents were monitored at 284 nm. The retention times were 2.5 min for Methotrexate and 5.0 min for Leflunomide. Linearity was observed in the ranges of 5–25 µg/mL and 4–20 µg/mL for Methotrexate and Leflunomide, respectively, with recoveries of 99.70–99.87% and 99.72–99.85%. Method validation confirmed the suitability of both UV and RP-HPLC techniques for the quantitative estimation of Methotrexate and Leflunomide in synthetic mixtures. **Conclusion:** ICH-compliant validation confirmed the applicability of the developed methods for the simultaneous analysis of Methotrexate and Leflunomide in synthetic mixtures.

INTRODUCTION:

Methotrexate is chemically 2S)-2-[(4-{[(4-amino-2-imino-2,3-dihydropteridin-6-yl) methyl](methyl)amino}phenyl)formamido]pentanedioi Acid. Methotrexate is an antimetabolite agent with anti-inflammatory properties. Methotrexate is a potent competitive inhibitor of enzyme dihydrofolate reductase. It is structurally similar to folic acid. Methotrexate may be used to treat rheumatoid arthritis, psoriasis, choriocarcinoma, childhood acute lymphoblastic leukaemia, Non-Hodgkin Lymphoma and bladder/breast cancer. Structure of Methotrexate showed in Figure 1[1-3].

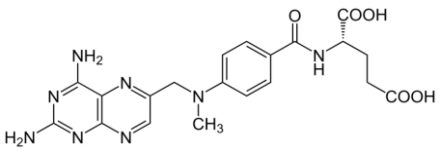

Figure 1: Structure of Methotrexate

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Leflunomide is chemically 5-methyl-N-[4-(trifluoromethyl) phenyl]-1,2-oxazole-4-carboxamide. Leflunomide is an immunosuppressive disease modifying antirheumatic drug (DMARD). Leflunomide is a pyrimidine synthesis inhibitor that works by inhibiting dihydroorotate dehydrogenase. Leflunomide used in active moderate to severe rheumatoid arthritis and psoriatic arthritis. Structure of Leflunomide showed in Figure 2[1-3].

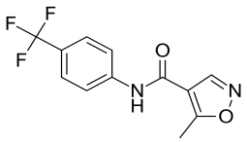

Figure 2: Structure of Leflunomide

Combination of Methotrexate adding in Leflunomide was studied under clinical trial phase was prove that synergistic effect in Patient with Psoriatic arthritis which lead to a reduction in bDMARD use and become cause effective as well as prevent Joints disease due to PsA[4].

Methotrexate and Leflunomide are listed in the Indian Pharmacopoeia (IP) 2022. A literature survey revealed that various analytical methods, including UV spectrophotometry, HPLC, HPTLC, GC, LC-MS, and LC/MS/MS[5-18], have been reported for the determination of these drugs individually or in combination with other drugs. However, no method has been documented for the simultaneous estimation of Methotrexate and Leflunomide. Therefore, the present study was undertaken to develop and validate a simple, accurate, and precise RP-HPLC method for their simultaneous estimation in a synthetic mixture.

MATERIALS AND METHODS:

Chemicals and Reagents:

Methotrexate was supplied as a bulk drug by JSK Chemicals, Ahmedabad, while Leflunomide was obtained as a gift sample from Centurion Healthcare Private Limited. All solvents were procured from Finar Chemicals, Ahmedabad, and AR-grade potassium dihydrogen phosphate was supplied by Astron Chemical Ltd., India. Fresh solutions were prepared daily.

Spectrophotometric and Chromatographic Condition:

The UV spectrophotometric analysis was performed using a Shimadzu UV-1800 spectrophotometer equipped with UV-Probe software, employing methanol as the solvent. Chromatographic analysis was carried out on a Systronics LC-138 system fitted with a photodiode array detector, manual injector, and a Kromstar Vertex C_{18} column (250 × 4.6 mm, 5 µm)[19,20]. Data acquisition and integration were accomplished using Clarify software. An isocratic mobile phase consisting of methanol and water (90:10 % v/v), with the pH adjusted to 2.5 using orthophosphoric acid, was used at a detection wavelength of 284 nm with a run time of 10 minutes. Under optimized chromatographic conditions, the retention times of Methotrexate and Leflunomide were found to be approximately 2.50 min and 5.00 min, respectively.

Method for Preparation of Analytical Solutions

Stock and standard Solution:

Accurately weigh 10 mg each of Methotrexate and Leflunomide and transfer to a volumetric flask. Add methanol and sonicate for 30 minutes to obtain a standard stock solution with a concentration of 100 µg/mL. Label the solution accordingly as the standard stock solution.

Preparation of Sample Solution:

An accurately weighed quantity of Methotrexate (25 mg) and Leflunomide (20 mg) was transferred to a 100 mL volumetric flask, and methanol was added up to the half mark. The solution was sonicated until complete dissolution of the drugs and then diluted to volume with methanol. The resulting solution was filtered through Whatman filter paper to obtain concentrations of 250 µg/mL for Methotrexate and 200 µg/mL for Leflunomide. From this solution, 0.4 mL was pipetted into a 10 mL volumetric flask and diluted to volume with methanol to yield final concentrations of 10 µg/mL and 8 µg/mL for Methotrexate and Leflunomide, respectively.

Preparation of Mobile phase:

A mixture of methanol and water in the ratio of 90:10 (% v/v) was prepared, thoroughly mixed, and the pH was adjusted to 2.5 using 10% orthophosphoric acid.

Selection of Suitable Analytical Wavelength:

The blank solution was scanned for absorbance over the range of 200–400 nm. The analytes were detected at a wavelength of 284 nm, at which both drugs exhibited satisfactory absorbance characteristics, as shown in Figure 3.

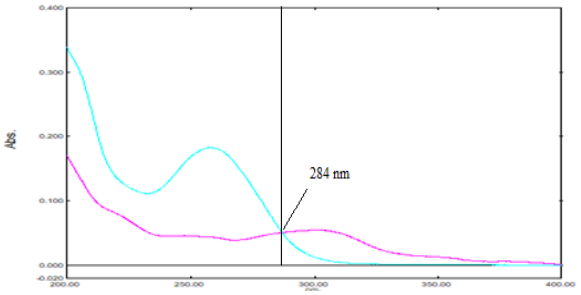

Figure 3: Overlain Zero order spectra of Methotrexate (10 µg/ml) and Leflunomide (8 µg/ml) in Methanol

UV-SPECTROPHOTOMETRIC AND RP-HPLC METHOD DEVELOPMENT AND VALIDATION

The objective of the present research was to develop a

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novel, reliable, practical, and cost-effective UV and RP-HPLC method for the simultaneous estimation of both drugs in a synthetic mixture. The developed method was validated in accordance with established guidelines for parameters including system suitability, linearity, precision, limits of detection and quantitation, accuracy, assay, and robustness

UV SPECTROPHOTOMETRIC METHOD

Selection Of Wavelength For Methotrexate And Leflunomide For wavelength selection, Methotrexate (10 µg/mL) and Leflunomide (8 µg/mL) solutions were scanned over the range of 200–400 nm. Methotrexate and Leflunomide exhibited maximum absorbance at λmax values of 232.74 nm and 267.09 nm, respectively, as depicted in Figure 4.

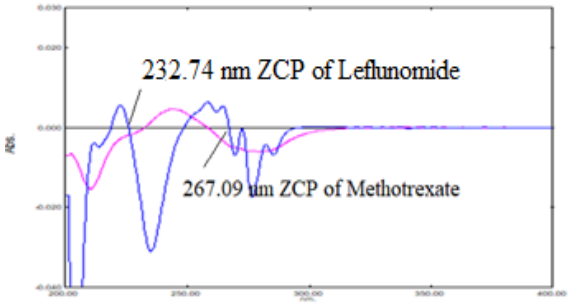

Figure 4: Overlay spectra of Methotrexate (10 µg/ml) and Leflunomide (8 µg/ml) in Methanol (First Order)

Linearity:

Accurately measured aliquots of Methotrexate stock solution (100 µg/mL) (0.5, 1.0, 1.5, 2.0, and 2.5 mL) and Leflunomide stock solution (100 µg/mL) (0.4, 0.8, 1.2, 1.6, and 2.0 mL) were transferred into five separate 10 mL volumetric flasks. The volumes were made up to the mark with methanol to obtain final concentrations of 5, 10, 15, 20, and 25 µg/mL for Methotrexate and 4, 8, 12, 16, and 20 µg/mL for Leflunomide. The absorbance of each solution was measured at 232.74 nm for Methotrexate and 267.09 nm for Leflunomide using methanol as the blank, and the results are depicted in Figures 5 and 6.[21]

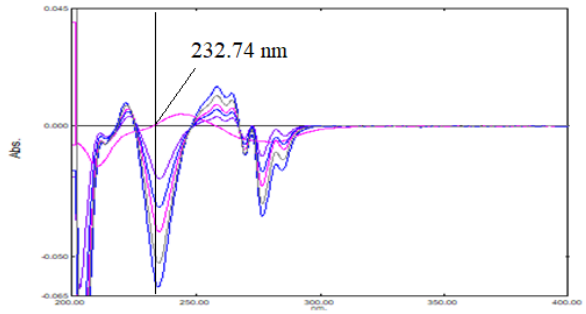

Figure 5: Linearity of 1st Derivative Spectra of Methotrexate (5-25 µg/ml) at 232.74 nm

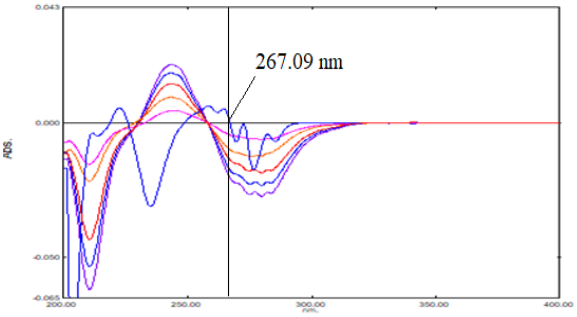

Figure 6: Linearity of 1st Derivative Spectra of Leflunomide (4-20 µg/ml) at 267.09 nm

Precision:

Intraday, Interday, and Repeatability studies were conducted to evaluate method precision. For the intraday study, Methotrexate solutions (5, 10, and 15 µg mL^{-1}) and Leflunomide solutions (4, 8, and 12 µg mL^{-1}) were analyzed in triplicate on the same day. Interday precision was assessed by analyzing the same concentration levels of Methotrexate and Leflunomide on three different days. Repeatability was evaluated by analyzing Methotrexate (10 µg mL^{-1}) and Leflunomide (8 µg mL^{-1}) six times. The results were expressed as percentage relative standard deviation (%RSD).[21]

Accuracy:

The pre-analyzed solution was spiked with known quantities of Methotrexate and Leflunomide at three concentration levels (50%, 100%, and 150%), and the mean percentage recovery for both drugs was calculated.[21]

Detection Limit and Quantification Limit:

In accordance with ICH guidelines, the Limits of Detection (LOD) and Quantification (LOQ) were calculated using standard equations.[21]

RP-HPLC METHOD DEVELOPMENT AND VALIDATION:

This study aimed to develop a simple, reliable, precise, and cost-effective RP-HPLC method for the simultaneous estimation of both drugs in a synthetic mixture. The proposed method was validated as per ICH guidelines for parameters including system suitability, linearity, precision, limits of detection and quantification, accuracy, assay, and robustness.

System Suitability:

System suitability testing was carried out by six replicate injections of freshly prepared standard solutions of Methotrexate and Leflunomide. Parameters including retention time, theoretical plates, and tailing factor were evaluated from the standard chromatogram, and the results are summarized in Table 1.

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Table 1: System Suitability Parameter

Name of drugs	Area	Retention time (min)	Tailing factor	No. of Theoretical Plates
Methotrexate	639.17	2.5	1.86	2036.57
Leflunomide	788.23	5	1.27	8238.79

Specificity:

To assess degradation and potential interferences, sample solutions of Methotrexate (10 µg mL^{-1}) and Leflunomide (8 µg mL^{-1}) were prepared and injected. The specificity of the method was confirmed by evaluating possible interference using a blank chromatogram, as well as individual and combined chromatograms of Methotrexate and Leflunomide, as depicted in Figures 7–10.

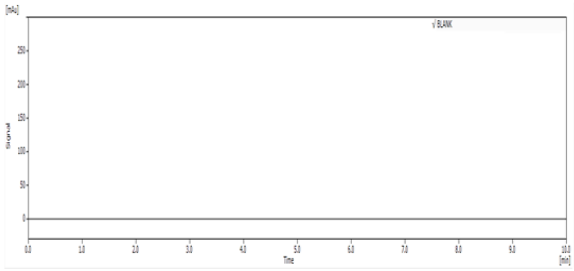

Figure 7: RP-HPLC Chromatogram of Blank in Methanol: Water (pH= 2.5) (90:10 %v/v) Flow rate: 1 ml/min at 284 nm

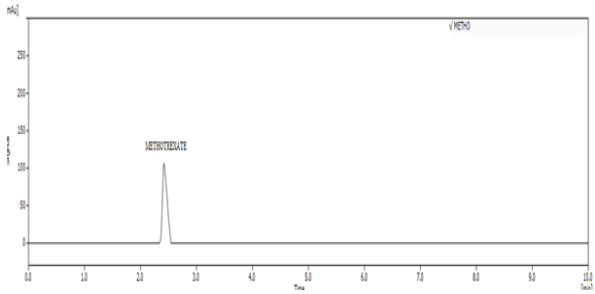

Figure 8: RP-HPLC Chromatogram of Methotrexate (10 µg/ml) in Methanol: Water (pH= 2.5) (90:10 %v/v) Flow rate: 1 ml/min at 284 nm

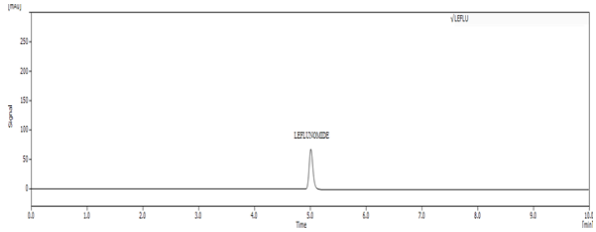

Figure 9: RP-HPLC Chromatogram of Leflunomide (8 µg/ml) in Methanol: Water (pH= 2.5) (90:10 %v/v) Flow rate: 1 ml/min at 284 nm

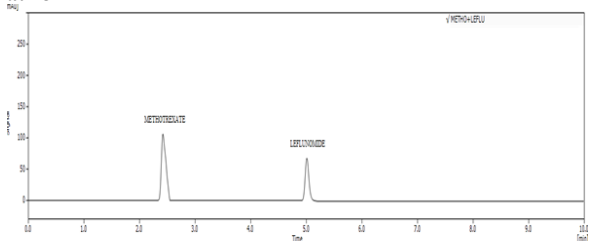

Figure 10: RP-HPLC Chromatogram of Methotrexate (10 µg/ml) and Leflunomide (8 µg/ml) in Methanol: Water (pH= 2.5) (90:10 %v/v) Flow rate: 1 ml/min at 284 nm

Linearity

Aliquots of the stock solution of Methotrexate (100 µg mL^{-1}), namely 0.5, 1.0, 1.5, 2.0, and 2.5 mL, and Leflunomide (100 µg mL^{-1}), namely 0.4, 0.8, 1.2, 1.6, and 2.0 mL, were pipetted into five separate 10 mL volumetric flasks. The solutions were diluted with the mobile phase [Methanol: Water (pH 2.5) (90:10% v/v)] to obtain final concentrations of 5, 10, 15, 20, and 25 µg mL^{-1} for Methotrexate and 4, 8, 12, 16, and 20 µg mL^{-1} for Leflunomide. A volume of 20 µL of each solution was injected into the RP-HPLC system using a Hamilton syringe, and the samples were analyzed. The results are depicted in Figure 11.[22]

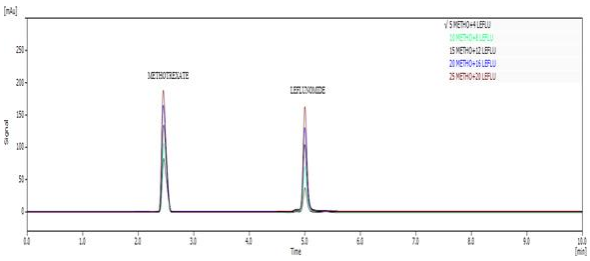

Figure 11: Overlay Chromatogram of Methotrexate (5 – 25 µg/ml) and Leflunomide (4 – 20 µg/ml) in Methanol: Water (pH= 2.5) (90:10 %v/v) Flow rate: 1 ml/min at 284 nm

Precision:

Intraday, Interday, and Repeatability studies were conducted to evaluate method precision. For the intraday study, Methotrexate solutions (5, 10, and 15 µg mL^{-1}) and Leflunomide solutions (4, 8, and 12 µg mL^{-1}) were analyzed in triplicate on the same day. Interday precision was assessed by analyzing the same concentration levels of Methotrexate and Leflunomide on three different days. Repeatability was evaluated by analyzing Methotrexate (10 µg mL^{-1}) and Leflunomide (8 µg mL^{-1}) six times. The results were expressed as percentage relative standard deviation (%RSD).[22]

Accuracy:

The pre-analyzed solution was spiked with known amounts of Bisoprolol fumarate and Trimetazidine HCl at three concentration levels 50%, 100%, and 150%. Each level was injected in triplicate into the HPLC system, and the mean percentage recovery for both drugs was calculated. [22]

Detection Limit and Quantification Limit:

According to ICH guidelines, the Detection Limit and Quantification Limit are calculated using standardized equations. [22]

Robustness:

Robustness was evaluated by deliberately introducing small, controlled variations in analytical parameters such as detection wavelength and flow rate, and verifying that the system suitability criteria were consistently met. Method robustness was further confirmed through repeated analysis under these

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modified conditions. [22]

RESULTS:

UV SPECTROPHOTOMETRIC METHHOD:

A reliable first order derivative Spectrophotometric method was developed for simultaneous estimation of Methotrexate and Leflunomide in synthetic mixture by UV Spectrophotometric.

Linearity:

The method demonstrated excellent linearity over the concentration ranges of 5–25 µg mL^{-1} for Methotrexate and 4–20 µg mL^{-1} for Leflunomide. The correlation coefficients were found to be 0.999 for Methotrexate and 0.997 for Leflunomide. The corresponding calibration curves are shown in Figures 12 and 13, and the detailed results are summarized in Table 2.

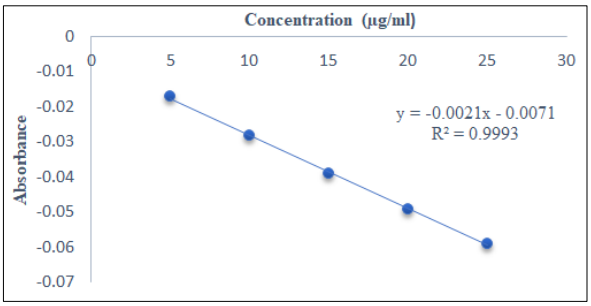

Figure 12: Calibration curve of Methotrexate (5 – 25) at 232.74 nm

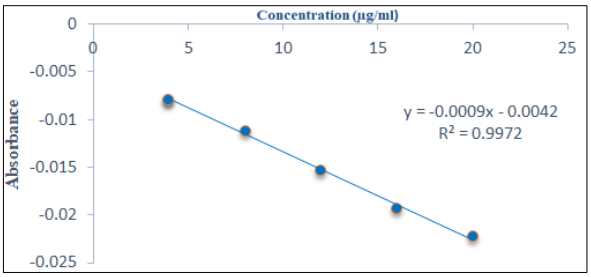

Figure 13: Calibration curve of Leflunomide (4 – 20 µg/ml) at 267.09 nm

Table 2: Linearity of Methotrexate and Leflunomide

Concentration (µg/ml)		Mean Absorbance ± SD (n=6)		% RSD	
Methotrexate	Leflunomide	Methotrexate	Leflunomide	Methotrexate	Leflunomide
5	4	|-0.0171 ± 0.00028|	|-0.0079 ± 0.00013|	1.57	1.74
10	8	|-0.0282 ± 0.00042|	|-0.0112 ± 0.00017|	1.41	1.55
15	12	|-0.0389 ± 0.00050|	|-0.0153 ± 0.00020|	1.29	1.35
20	16	|-0.0493 ± 0.00057|	|-0.0193 ± 0.00021|	1.16	1.12
25	20	|-0.0589 ± 0.00066|	|-0.0223 ± 0.00022|	1.12	1.02

Precision:

Precision refers to the closeness of agreement among a series of measurements obtained from multiple samplings of the same homogeneous sample. Method precision was evaluated through intraday, interday, and repeatability studies. The %RSD values for system precision are presented in Tables 3 and 4 for Methotrexate and Leflunomide, respectively. Since all %RSD values were below 2%, the method was confirmed to be precise, reproducible, and repeatable.

Table 3: Precision Study of Methotrexate

Intraday Precision of Methotrexate		
Conc. (µg/ml)	Mean Area ± SD (n=3)	% RSD
5	|-0.0172 ± 0.00025|	1.47
10	|-0.0289 ± 0.00040|	1.38
15	|-0.0392 ± 0.00049|	1.26
Interday Precision of Methotrexate		
Conc. (µg/ml)	Mean Area ± SD (n=3)	% RSD
5	|-0.0167 ± 0.00026|	1.59
10	|-0.0287 ± 0.00037|	1.30
15	|-0.0392 ± 0.00043|	1.11
Repeatability of Methotrexate		
Conc. (µg/ml)	Mean Area ± SD (n=6)	% RSD
10	|-0.0285 ± 0.00028|	0.96

Table 4: Precision Study of Leflunomide

Intraday Precision of Leflunomide		
Conc. (µg/ml)	Mean Area ± SD (n=3)	% RSD
4	|-0.0080 ± 0.00012|	1.50
8	|-0.0112 ± 0.00014|	1.25
12	|-0.0148 ± 0.00015|	1.01
Interday Precision of Leflunomide		
Conc. (µg/ml)	Mean Area ± SD (n=3)	% RSD
4	|-0.0080 ± 0.00012|	1.50
8	|-0.0112 ± 0.00014|	1.25
12	|-0.0148 ± 0.00015|	1.01
Repeatability of Leflunomide		
Conc. (µg/ml)	Mean Area ± SD (n=6)	% RSD
8	|-0.0111 ± 0.00010|	0.92

Accuracy:

Recovery studies were conducted at three concentration levels (50%, 100%, and 150%). Three replicates at each level were analyzed, and the mean percentage recoveries were calculated. As shown in Table 5, the recovery values for Methotrexate and Leflunomide ranged from 99.48% to 99.68% and 99.69% to 99.75%, respectively. Since all recovery values were within the acceptable range of 98.0%–102%, the method was confirmed to be accurate. These satisfactory recovery results further demonstrate the suitability of the method for routine quality control analysis.

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Table 5: Recovery of Methotrexate and Leflunomide

Name of Drug	%Level Of Recovery	Test Amount (µg/ml)	Amount of drug taken (µg/ml)	Spiked Std Amount (µg/ml)	Total amount Recovered (µg/ml)	% Recovery ±S.D(n=3)
Methotrexate	50	10	5	15	14.91	99.48 ±0.1708
	100	10	10	20	19.90	99.51 ±0.3013
	150	10	15	25	24.91	99.68 ±0.5256
Leflunomide	50	8	4	12	11.95	99.69 ±0.2993
	100	8	8	16	15.95	99.72 ±0.3671
	150	8	12	20	19.970	99.75 ±0.4070

Detection Limit and Quantitation Limit

The Limit of Detection (LOD) indicates the lowest concentration of analyte that can be detected, while the Limit of Quantification (LOQ) represents the lowest concentration that can be quantified with acceptable accuracy and precision, making it useful for the assessment of impurities or degradation products. As shown in Table 6, the LOD and LOQ values were 0.035 µg mL^{-1} and 0.115 µg mL^{-1} for Methotrexate, and 0.220 µg mL^{-1} and 0.726 µg mL^{-1} for Leflunomide, respectively.

Table 6: LOD and LOQ for Methotrexate and Leflunomide

Parameter	Methotrexate	Leflunomide
LOD(µg/ml)	0.035	0.220
LOQ(µg/ml)	0.115	0.726

Assay:

Three replicate injections of the same sample solution were analyzed, and the resulting chromatograms were recorded. Methotrexate and Leflunomide showed mean recoveries of 99.03% and 99.04%, respectively, as presented in Table 7.

Table 7: Analysis of Pharmaceutical Dosage form

Name of Drug	Amount taken (µg/ml)	amount Found(µg/ml)	%Assay ± S.D (n=3)	% RSD
Methotrexate	10	9.92	99.03 ± 0.6110	0.616
Leflunomide	8	7.92	99.04 ± 0.5022	0.507

RP-HPLC METHOD:

An isocratic RP-HPLC method was successfully developed and validated for the simultaneous estimation of Methotrexate and Leflunomide in a synthetic mixture. The method is simple, rapid, accurate, and precise. Both analytes showed maximum absorbance at 284 nm, which was selected as the detection wavelength. Optimal chromatographic performance was achieved by appropriate selection of flow rate and detection wavelength. Efficient separation with well-resolved peaks was obtained using a mobile phase of Methanol: Water (pH 2.5) in the ratio of 90:10 % v/v at a flow rate of 1.0 mL min^{-1}. A Kromstar Vertex C_{18} column (250 × 4.6 mm, 5 µm) was used as the stationary phase at ambient temperature with an injection volume of 20 µL, ensuring good reproducibility and repeatability.

Linearity

The method demonstrated excellent linearity over the concentration ranges of 5–25 µg mL^{-1} for Methotrexate and 4–20 µg mL^{-1} for Leflunomide. The correlation coefficients were found to be 0.996 for Methotrexate and 0.996 for Leflunomide. The corresponding calibration curves are shown in Figures 14 and 15, and the detailed results are summarized in Table 8.

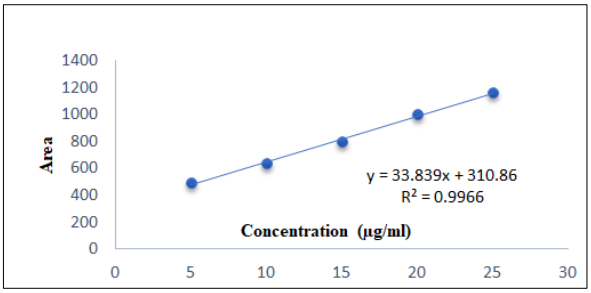

Figure 14: Calibration curve of Methotrexate (5 – 25) at 284 nm

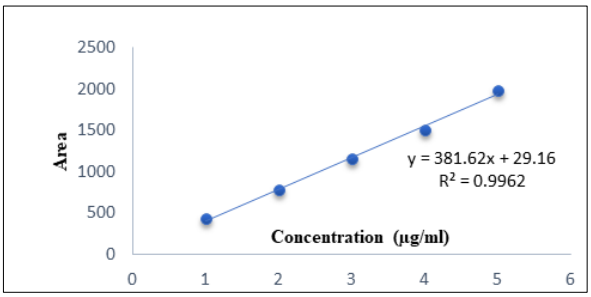

Figure 15: Calibration curve of Leflunomide (4 – 20 µg/ml) at 284 nm

Table 8: Linearity of Methotrexate and Leflunomide

Concentration (µg/ml)		Area ± SD (n=6)		% RSD	
Methotrexate	**Leflunomide**	**Methotrexate**	**Leflunomide**	**Methotrexate**	**Leflunomide**
5	4	497.94 ± 7.648	436.50 ± 6.477	1.57	1.48
10	8	639.17 ± 9.038	788.23 ± 10.761	1.41	1.37
15	12	789.86 ± 10.184	1155.97 ± 14.266	1.28	1.23
20	16	1015.83 ± 11.980	1507.95 ± 17.079	1.17	1.13
25	20	1150.81 ± 12.036	1984.74 ± 20.944	1.05	1.05

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Precision:

Precision refers to the closeness of agreement among a series of measurements obtained from multiple samplings of the same homogeneous sample. Method precision was evaluated through intraday, interday, and repeatability studies. The %RSD values for system precision are presented in Tables 9 and 10 for Methotrexate and Leflunomide, respectively. Since all %RSD values were below 2%, the method was confirmed to be precise, reproducible, and repeatable.

Table 9: Precision Study of Methotrexate

Intraday Precision of Methotrexate		
Conc. (µg/ml)	**Mean Area ± SD (n=3)**	**% RSD**
5	494.36 ± 6.183	1.25
10	641.77 ± 7.165	1.12
15	790.34 ± 7.845	0.99
Interday Precision of Methotrexate		
Conc. (µg/ml)	**Mean Area ± SD (n=3)**	**% RSD**
5	494.25 ± 6.431	1.30
10	640.77 ± 7.99	1.24
15	790.496 ± 8.34	1.05
Repeatability of Methotrexate		
Conc. (µg/ml)	**Mean Area ± SD (n=6)**	**% RSD**
10	636.50 ± 6.104	0.96

Table 10: Precision Study of Leflunomide

Intraday Precision of Leflunomide		
Conc. (µg/ml)	**Mean Area ± SD (n=3)**	**% RSD**
4	436.40 ± 6.022	1.38
8	787.86 ± 9.784	1.24
12	1137.30 ± 11.417	1.00
Interday Precision of Leflunomide		
Conc. (µg/ml)	**Mean Area ± SD (n=3)**	**% RSD**
4	437.60 ± 6.104	1.39
8	792.23 ± 10.291	1.29
12	1136.63 ± 11.842	1.04
Repeatability of Leflunomide		
Conc. (µg/ml)	**Mean Area ± SD (n=6)**	**% RSD**
8	789.73 ± 7.807	0.98

Accuracy:

Recovery studies were conducted at three concentration levels (50%, 100%, and 150%). Three replicates at each level were analyzed, and the mean percentage recoveries were calculated. As shown in Table 11, the recovery values for Methotrexate and Leflunomide ranged from 99.70% to 99.87% and 99.72% to 99.85%, respectively. Since all recovery values were within the acceptable range of 98.0%–102%, the method was confirmed to be accurate. These satisfactory recovery results further demonstrate the suitability of the method for routine quality control analysis.

Table 11: Recovery of Methotrexate and Leflunomide

Name of Drug	%Level Of Recovery	Test Amount (µg/ml)	Amount of drug taken (µg/ml)	Spiked Std Amount (µg/ml)	Total amount Recovered (µg/ml)	% Recovery ±S.D (n=3)
Methotrexate	50	10	5	15	15.04	99.70 ± 0.3064
	100	10	10	20	20.05	99.73 ± 0.4446
	150	10	15	25	25.03	99.87 ± 0.5303
Leflunomide	50	8	4	12	11.98	99.72 ± 0.3101
	100	8	8	16	15.97	99.73 ± 0.4157
	150	8	12	20	19.97	99.85 ± 0.5242

Detection Limit and Quantitation Limit:

The Limit of Detection (LOD) indicates the lowest concentration of analyte that can be detected, while the Limit of Quantification (LOQ) represents the lowest concentration that can be quantified with acceptable accuracy and precision, making it useful for the assessment of impurities or degradation products. As shown in Table 12, the LOD and LOQ values were 0.455 µg mL^{-1} and 1.50 µg mL^{-1} for Methotrexate, and 0.482 µg mL^{-1} and 1.59 µg mL^{-1} for Leflunomide, respectively.

Table 12: LOD and LOQ for Methotrexate and Leflunomide

Parameter	Methotrexate	Leflunomide
LOD(µg/ml)	0.455	0.482
LOQ(µg/ml)	1.50	1.59

Robustness:

Deliberate variations in flow rate and detection wavelength were introduced, and the results are summarized in Table 13. The findings indicated that these minor changes did not significantly affect the analytical performance, thereby confirming the robustness of the method.

Table 13: Robustness data for Methotrexate and Leflunomide

SR NO.	Parameter	Variation	Area ± S.D (n=3)		% RSD	
			Methotrexate	**Leflunomide**	**Methotrexate**	**Leflunomide**
1	Flow rate (1 ml/min) (±0.2 ml/min)	0.8 ml/min	637.39 ± 4.451	793.43 ± 7.258	0.69	0.91
2		**1.0 ml/min**	**636.01 ± 2.670**	**793.49 ± 5.990**	**0.41**	**0.75**
3		1.2 ml/min	636.49 ± 3.715	793.88 ± 6.751	0.58	0.85
1	Detection Wavelength (284 nm) (± 2 nm)	282 nm	637.32 ± 4.463	794.04 ± 6.249	0.70	0.78
2		**284 nm**	**636.01 ± 2.681**	**795.23 ± 4.544**	**0.42**	**0.57**
3		286 nm	634.22 ± 3.884	794.15 ± 5.532	0.61	0.69

Assay:

Three replicate injections of the same sample solution were analyzed, and the resulting chromatograms were recorded. Methotrexate and Leflunomide showed

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mean recoveries of 99.11% and 99.49%, respectively, as presented in Table 14.

Table 14: Analysis of Pharmaceutical Dosage form

Name of Drug	Amount taken (µg/ml)	amount Found(µg/ml)	%Assay ± S.D (n=3)	% RSD
Methotrexate	10	9.8	99.11 ± 0.6235	0.629
Leflunomide	8	7.8	99.49 ± 0.5643	0.567

DISCUSSION:

The method was carefully optimized to improve sensitivity and specificity. Validation in accordance with ICH guidelines demonstrated satisfactory linearity, precision, accuracy, and robustness. Future studies may evaluate its applicability to various pharmaceutical formulations and dosage forms.

CONCLUSION:

The results of the present study confirm that the proposed UV spectrophotometric and RP-HPLC methods are simple, rapid, accurate, and cost-effective for the simultaneous estimation of Methotrexate and Leflunomide in a synthetic mixture. Statistical evaluation demonstrated excellent repeatability, precision, and selectivity, satisfying all ICH validation requirements. The methods exhibited robust performance under deliberate variations in chromatographic conditions, indicating high reliability for routine laboratory application.

Furthermore, the high recovery values and low %RSD substantiate the suitability of the methods for quality control analysis and their ability to detect minor variations in drug concentration. The acceptable LOD and LOQ values also highlight the sensitivity of the methods, supporting their potential use in impurity profiling and stability-indicating studies.

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CONFLICT OF INTEREST:

The authors declare that there is no conflict of interest.

ABBREVIATIONS:

ICH: International Council for Harmonization; **UV:** Ultraviolet, **RP-HPLC:** Reverse phase High Performance liquid chromatography; **API:** Active Pharmaceutical Ingredient; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **RSD:** Relative Standard deviation.

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