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Research Article

DEVELOPMENT AND VALIDATION OF ANALYTICAL METHOD FOR SIMULTANEOUS ESTIMATION OF LEFLUNOMIDE AND METHOTREXATE IN SYNTHETIC MIXTURE BY Q-ABSORBANCE RATIO METHOD

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Abstract

The present work involves simultaneous estimation of Leflunomide and Methotrexate in synthetic mixture by UV Spectrophotometric method. Leflunomide has an absorbance maximum at 259 nm and Methotrexate has two absorbance maxima at 259 nm and 303 nm in methanol: water (1:1) mixture. For Q absorbance ratio method, Absorbance at isoabsorptive point 286nm and at 259nm was selected. Both the drugs and their mixture obey Beers and Lamberts law at selected wavelength. The linearity was observed in the concentration range 5-25 µg/ml for Leflunomide and 4-20 µg/ml for Methotrexate. The result of analysis has been validated statistically and recovery studies confirmed the accuracy of the proposed method. The proposed procedures are simple, rapid and economical can be used for the routine analysis of both drugs.

Keywords: Method development, Validation, Q-Absorbance Ratio, Leflunomide, Methotrexate.

Introduction:

Methotrexate ((2S) 2[(4{[(2,4diaminopteridin6yl)methyl] (methyl)aminophenyl) formamido] pentanedioicacid) [1,2,3] an antimetabolite that inhibits purine pathways, has been the hallmark of standard of care of many years in the rheumatoid arthritis(RA)[3,4], However many RA patients continue to have active disease despite maximal doses of Methotrexate. In contrast to Methotrexate, Leflunomide is chemically (5-methyl-N-[4-(trifluoromethyl) phenyl]-1,2-oxazole-4-carboxamide) [1,2,3]. It is a DMARD (disease-modifying anti-rheumatic drug) that inhibits pyrimidine pathways. Both the drugs are official in I.P, B.P, and U.S.P.

Recent studies indicate that combinations of disease-modifying anti-rheumatic drug (DMARD) therapy can provide improved clinical benefit for those patients who continue to have active disease despite Methotrexate[4]. Combination therapy of Leflunomide with Methotrexate suggests a possible alternative for those patients with persistent active RA who fail Methotrexate monotherapy[4].

Several UV[6,8,9], HPLC[5,7,10]methods are reported in combination with other drugs for the determination of Methotrexate and Leflunomide in the literature for its assay. However, no method is reported for simultaneous estimation of Methotrexate and Leflunomide by UV Spectrophotometric method in any literature. In the present investigation, a simple, precise and accurate method is described for the simultaneous estimation of these two drugs.

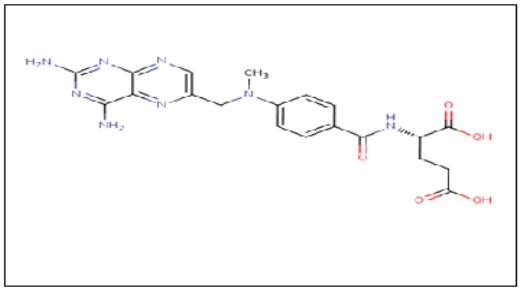

Figure 1: Structure of Methotrexate

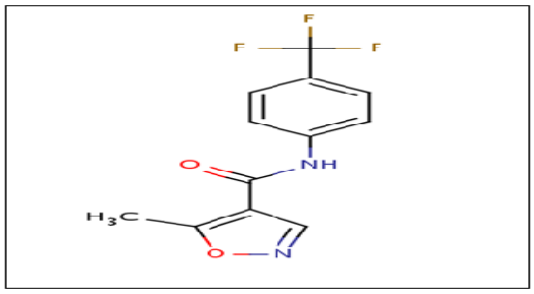

Figure 2: Structure of Leflunomide

Material and Methods

Instrumentation

Spectrophotometric measurements were performed on Shimadzu UV visible double beam spectrophotometer (Model-1800). All weighing were done on electronic analytical balance (Wensar Dab 220).

Chemicals and Reagents

The bulk drug Methotrexate obtained from West coast Pharmaceuticals, Ahmedabad. Leflunomide obtained from Stellar Chemical Laboratories Derol, Panchmahal. Analytical grade methanol was procured from Merck Fine chemicals (Mumbai).

Selection of a Solvent

Methanol: Water (1:1) was selected as solvent for studying spectral characteristic of drugs.

Preparation of Standard Solution

(A) Preparation of Standard Solution of Leflunomide

Preparation of Standard Stock Solution of Leflunomide (100µg/ml)

Accurately weighed quantity of LEF 10 mg was transferred to 100 ml volumetric flask, dissolved in 10 ml of Methanol: Water (1:1) and diluted up to mark with Methanol: Water (1:1) to give a stock solution having strength of 100µg/ml.

Preparation of Working Standard Solution of Leflunomide

From the above stock solution pipette out 0.5 mL, 1.0mL, 1.5mL, 2.0mL, and 2.5mL of solution and transferred to 10 mL volumetric flask and make up the volume up to 10 mL with methanol: Water (1:1) to Produce concentration 5, 10, 15, 20 and 25 µg/mL respectively.

B) Preparation of Standard Solution of Methotrexate

Preparation of Standard Stock Solution of Methotrexate (100µg/ml)

Accurately weighed quantity of MTX 10 mg was transferred to 100 ml volumetric flask, dissolved in 10 ml of Methanol: Water (1:1) and diluted up to mark with Methanol: Water (1:1) to give a stock solution having strength of 100µg/ml.

Preparation of Working Standard Solution of Methotrexate

From the above stock solution pipette out 0.4 mL, 0.8 mL, 1.2 mL, 1.6 mL, and 2mL of solution and transferred to 10 mL volumetric flask and make up the volume up to 10 mL with methanol: Water (1:1) to Produce concentration 4, 8, 12, 16 and 20 µg/mL respectively.

C) Preparation of synthetic mixture of Leflunomide and Methotrexate

The synthetic mixture of Leflunomide and Methotrexate was prepared in the ratio of 4:1.Accurately weighed Leflunomide (10 mg) and Methotrexate (2.5mg) were transferred in 100 mL volumetric flask and dissolved in methanol: water (1:1) (70 mL). Common excipients, which are used in the tablet formulation, were added in this mixture and sonicated for 20 minutes. This solution was filtered through the Whatmann filter paper No. 41 and the residue was

washed thoroughly with methanol: water (1:1). The filtrate and washings were combined and diluted to the mark with methanol: water (1:1) to get solution having Leflunomide(100 µg/mL) and Methotrexate (25 µg/mL).

Selection of Analytical Wavelength

To determine wavelength for measurement, standard spectra of MTX and LEF were scanned between 200-400 nm against Methanol: Water (1:1). Absorbance maxima were obtained at 259 nm and at 302 nm for LEF and MTX respectively and Iso-absorptive point were obtained at 286 nm.

Preparation of Calibration Curve

(A)Calibration Curve for Leflunomide

Calibration curve for LEF consists of different concentrations of standard LEF solution ranging from 5-25 µg/ml. The solutions were prepared by pipetting out 0.5, 1.0, 1.5, 2.0 and 2.5 ml of the working standard solution of LEF (100µg/ml) into series of 10 ml volumetric flasks and the volume was adjusted to mark with Methanol: Water (1:1). The absorbance of the solutions was measured at 259 nm and 286 nm against Methanol: Water (1:1) as a blank. Calibration curve was plotted at both wavelengths and two equations were formed using the absorptivity.

(B) Calibration Curve for Methotrexate

Calibration curve for MTX consists of different concentrations of standard MTX solution ranging from 4 – 20 µg/ml. The solutions were prepared by pipetting out 0.4, 0.8, 1.2, 1.6 and 2.0 ml of the working standard solution of MTX (100µg/ml) into series of 10 ml volumetric flasks and the volume was adjusted to mark with Methanol: Water (1:1). The absorbance of the solutions was measured at 259 nm and 286 nm against Methanol: Water (1:1) as a blank. Calibration curve was plotted at both wavelengths and two equations were formed using the absorptivity.

Preparation of Sample solution

About 5.0 mg of Synthetic mixture was weighed accurately and transferred into a 50 mL volumetric flask. The content was mixed with Methanol: Water (1:1) (70 ml) and sonicated for 20 min to dissolve the drug as completely as possible. The solution was then filtered through a Whatman filter paper no. 41. The volume was adjusted up to mark with Methanol: Water (1:1). The mixture contain 100µg/ml of Leflunomide and 25µg/ml of Methotrexate .An aliquot of this solution (2 ml) was transferred in to a 10 ml volumetric flask and the volume was adjusted up to the mark with Methanol: Water (1:1) to make final concentration of Leflunomide (10 µg/ml) and Methotrexate (2.5 µg/ml)

Validation [11]

Linearity and Range

The linearity response was determined by analyzing 5 independent levels of calibration curve in the range of 5-25 µg/ml and 4-20 µg/ml for LEF and MTX respectively (n = 5).

The calibration curve of absorbance vs. respective concentration was plotted and correlation coefficient and regression line equations for CEF and MTX were calculated.

Precision

(A)Repeatability

Aliquots of 1.5ml of working standard solution of LEF (100 µg/ml) were transferred to a 10 ml volumetric flask. Aliquots of 1.2ml of working standard solution of MTX (100 µg/ml) were respectively transferred to a 10 ml volumetric flask. The volume was adjusted up to mark with methanol to get 15µg/ml solution of LEF and 12µg/ml solution of MTX. The absorbance of solution was measured six times and % RSD was calculated.

(B) Intraday precision

Aliquots of 1.0, 1.5, and 2.0 ml of working standard solution of LEF (100 µg/ml) were transferred to a series of 10 ml volumetric flask. Aliquots of 0.8, 1.2 and 1.6 ml of working standard solution of MTX (100 µg/ml) were respectively transferred to the same series of 10 ml volumetric flask. The volume was adjusted up to mark with methanol: Water (1:1) to get 10, 15 and 20µg/ml solution of LEF and 8, 12 and 16µg/ml solution of MTX. Solution was analyzed 3 times on the same day and % RSD was calculated.

(C) Interday Precision

Aliquots of 1.0, 1.5, and 2.0 ml of working standard solution of LEF (100 µg/ml) were transferred to a series of 10 ml volumetric flask. Aliquots of 0.8, 1.2 and 1.6 ml of working standard solution of MTX (100 µg/ml) were respectively transferred to the same series of 10 ml volumetric flask. The volume was adjusted up to mark with methanol: Water (1:1) to get 10, 15 and 20µg/ml solution of LEF and 8, 12 and 16µg/ml solution of MTX. Solution was analyzed 3 times on the 3 different days and % RSD was calculated.

Limit of Detection (LOD)

The LOD is estimated from the set of 5 calibration curves used to determine method linearity.

The LOD may be calculated as,

$LOD = 3.3 * SD/Slope$

Where, SD = the standard deviation of Y- intercept of 5 calibration curves.

Slope = the mean slope of the 5 calibration curves.

Limit of Quantification (LOQ)

The LOQ is estimated from the set of 5 calibration curves used to determine method linearity.

The LOD may be calculated as,

$LOQ = 10 * SD/Slope$

Where, SD = the standard deviation of Y- intercept of 5 calibration curves.

Slope = the mean slope of the 5 calibration curves.

Accuracy

The accuracy of the method was determined by calculating recovery of LEF and MTX by the standard addition method. Aliquots of 0.8, 1.0, and 1.2 ml of working standard solution of LEF(100 µg/ml) were added at 80, 100 and 120 % level to pre-analyzed 1.0 ml sample solutions of LEF and MTX (100 µg/mL of LEF and MTX) transferred to a series of 10 ml volumetric flask. The volume was adjusted up to mark with methanol to get 18, 20 and 22µg/ml solution of LEF.

Aliquots of 0.2, 0.25, and 3.0 ml of working standard solution of MTX (100 µg/ml) were added at 80, 100 and 120 % level to pre-analyzed 1 ml sample solutions of LEF and MTX (100 µg/ mL of LEF and MTX) transferred to a series of 10 ml volumetric flask. The volume was adjusted up to mark with methanol to get 4.5, 5 and 5.5µg/ml solution of MTX. Absorbance of solution was measured at selected wavelengths for LEF and MTX. The amount of LEF and MTX was calculated at each level and % recoveries were calculated by measuring the absorbance and fitting the values in equation. Accuracy was assessed using three concentrations and three replicates of each.

Q-Absorbance Ratio Method

• Absorbance ratio method uses the ratio of absorbance at two selected wavelengths, one which is an Iso-absorptive point and other being the λ max of one of the two components.

• From the overlay spectra of two drugs, it is evident that LEF and MTX show an Iso-absorptive point at 286 nm. The second wavelength used is 259 nm, which is λ max of LEF

• Five working standard solutions having concentration 5, 10, 15, 20 and 25 µg/mL for LEF and 4, 8, 12, 16 and 20 µg/mL for MTX were prepared in methanol: Water (1:1) and the absorbance at 286 nm (Iso-absorptive point) and 259 nm (λ max of LEF) were measured and absorptivity coefficients were calculated.

• The absorbance of the sample solution (20 µg/ml of LEF and 5 µg/ml of MTX) i.e. A_1 and A_2 were recorded at 286 nm (Iso-absorptive point) and 259 nm (λ max of LEF) respectively, and ratios of absorbance were calculated, i.e. A_2/A_1

• Relative concentration of two drugs in the sample was calculated using following equations.

$C_X = [(Q_M-Q_Y)/ (Q_X-Q_Y)] \times A_1/ax_1$…….. (iii)

$C_Y = [(Q_M-Q_X)/ (Q_Y-Q_X)] \times A_1/ay_1$…….. (iv)

The Q-values and absorptivity for both drugs were calculated as follows,

Q_M = Absorbance of Sample solution at 259 nm (A_2)/ Absorbance of Sample solution at 286 nm (A_1)

Q_X = Absorptivity of LEF at 259 nm (ax_2)/ Absorptivity of LEF at 286nm (ax_1)

Q_Y = Absorptivity of MTX at 259 nm (ay_2)/ Absorptivity of MTX at 286 nm (ay_1)

Where,

A_1 and A_2 are absorbance of mixture at 286 nm and 259 nm

QX And QY are Q value of LEF and MTX respectively

ax_1 and ay_1 are absorptivity of LEF and MTX at 286 nm

ax_2 and ay_2 are absorptivity of LEF and MTX at 259 nm

The analysis procedure was repeated 3 times with sample solution.

Results and Discussion

A reliable Q absorption ratio method was developed for simultaneous estimation of Leflunomide and Methotrexate in synthetic mixture by UV Spectrophotometry. Beers law was obeyed in concentration range of 5-25 µg/ml for Leflunomide and 4-20 µg/ml for methotrexate at 286 nm and 259 nm wavelengths. The correlation coefficient Leflunomide and Methotrexate was found to be R^2 = 0.999 and 0.998. The mean % recoveries were found to be in the range of 99.15- 99.56% and 99.08 -102.4% for Leflunomide and Methotrexate respectively. The LOD and LOQ were 0.257µg/ml and 0.716µg/ml of Leflunomide 0.098µg/ml and 0.299µg/ml of Methotrexate, respectively. The proposed

method was precise, accurate and reproducible and acceptable recovery of the analyte, which can be applied for the analysis of Leflunomide and Methotrexate in Synthetic Mixture.

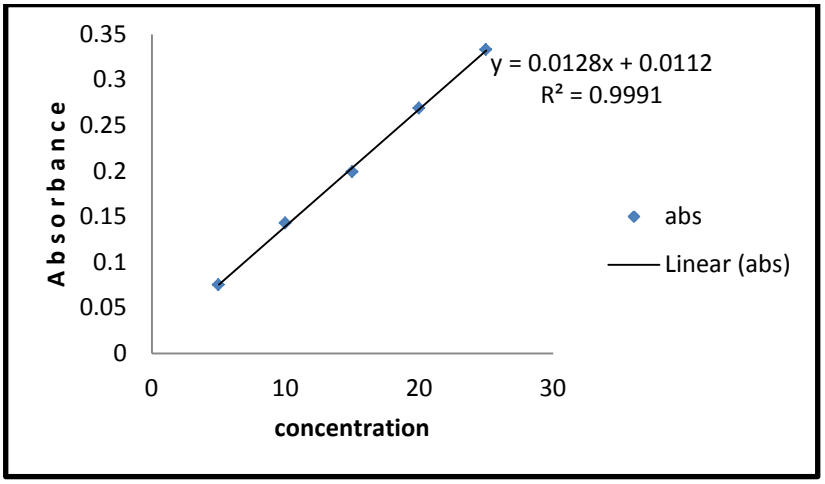

Figure-3: Calibration curve of Leflunomide at 286nm.

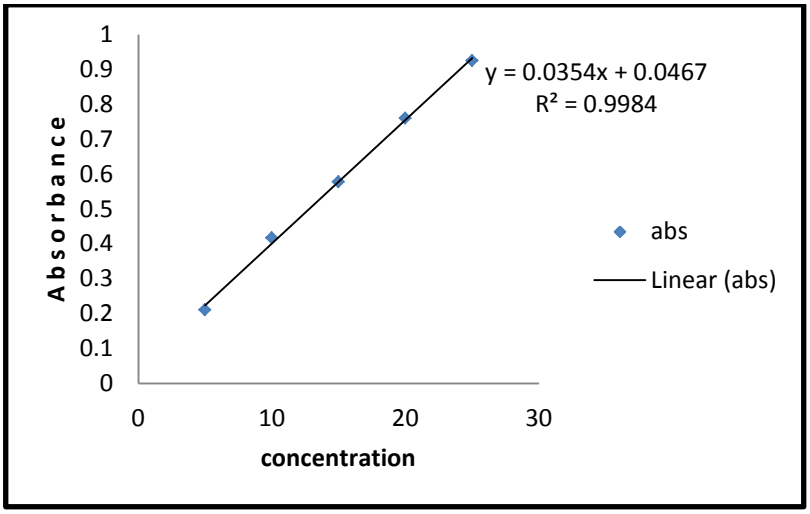

Figure-4: Calibration curve of Leflunomide at 259 nm.

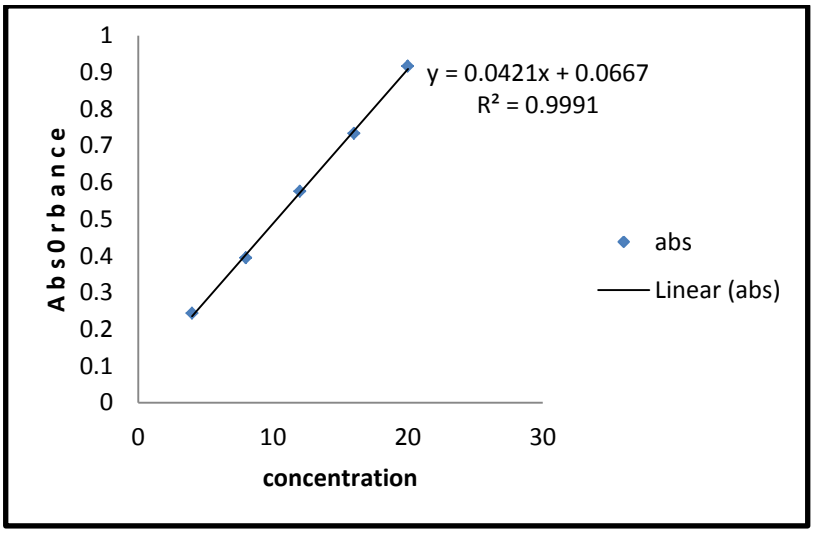

Figure-5: Calibration curve of Methotrexate at 286 nm.

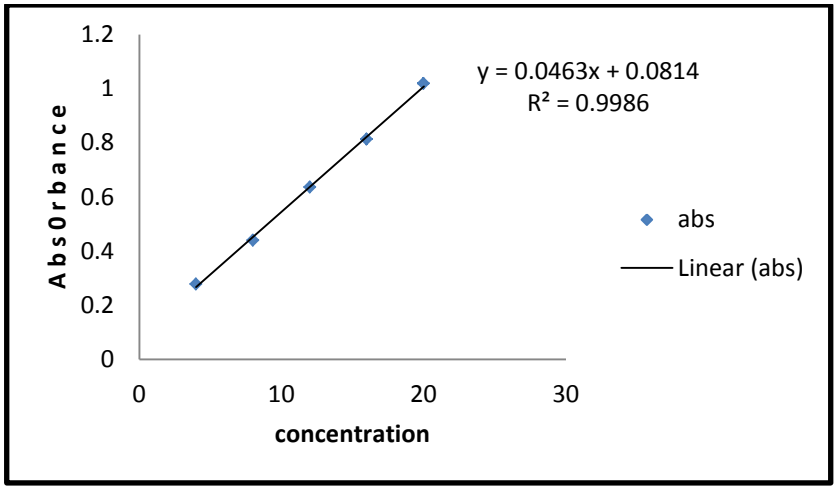

Figure-6: Calibration curve of Methotrexate at 259 nm.

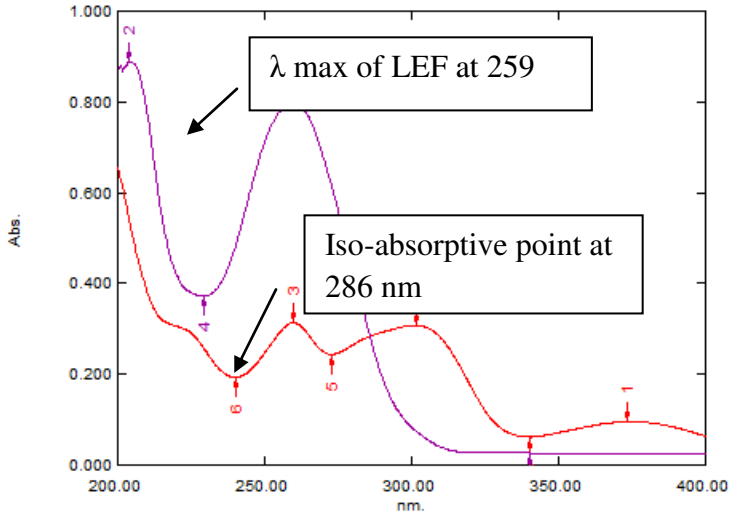

Figure-7: Overlay spectra of Methotrexate (5µg/ml) and Leflunomide (20 µg/ml).

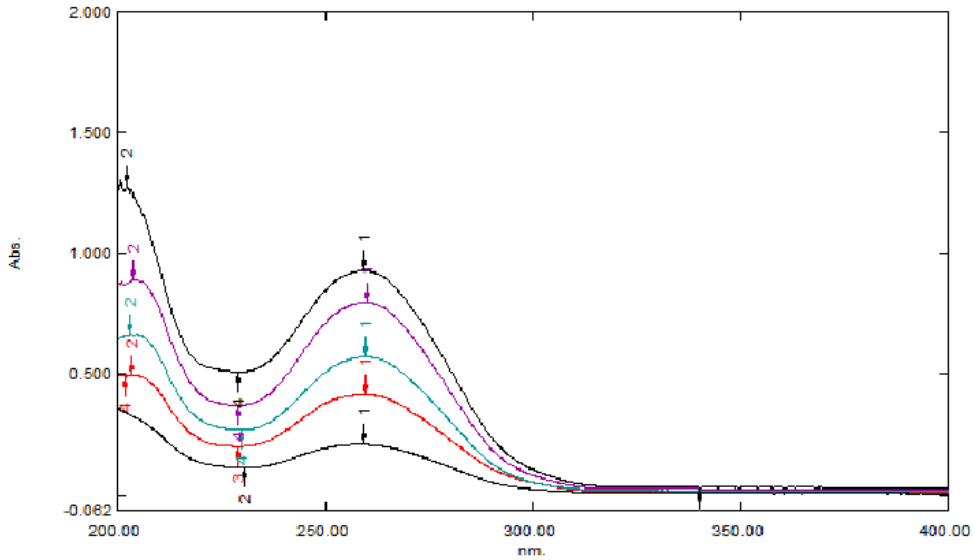

Figure-8: Overlay spectra of Leflunomide (5-25 µg/ml).

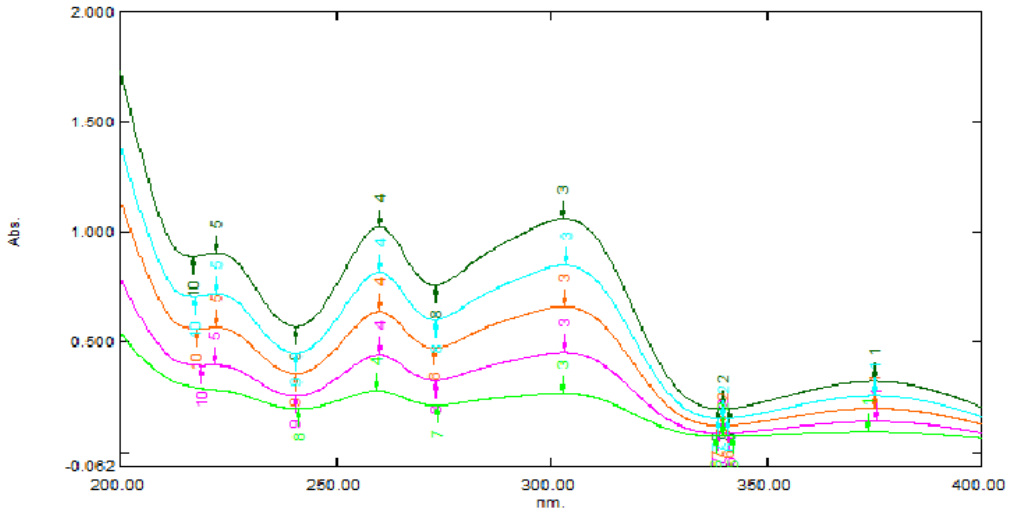

Figure-9: Overlay spectra of Methotrexate (4-20 µg/ml).

Table-1: Linearity data of Leflunomide.

AT 286 nm			At 259 nm		
Con (µg/ml)	**Mean absorbance ±SD(n=5)**	**%RSD**	**Con (µg/ml)**	**Mean absorbance ±SD (n=5)**	**%RSD**
5	0.074 ± 0.0054	0.7351	5	0.211 ± 0.00089	0.4238
10	0.144 ±0.00098	0.6819	10	0.416 ± 0.0008	0.196
15	0.198 ± 0.00098	0.4961	15	0.576 ± 0.00098	0.170
20	0.268 ±0.00051	0.1920	20	0.760 ± 0.00075	0.099
25	0.332±0.00075	0.2260	25	0.925 ± 0.0006	0.068

Table-2: Linearity data of Methotrexate.

AT 286 nm			At 259 nm		
Con (µg/ml)	**Mean absorbance ±SD (n=5)**	**%RSD**	**Con (µg/ml)**	**Mean absorbance ±SD (n=5)**	**%RSD**
4	0.242333±0.00081	0.336931	4	0.278167±0.0011	0.420268
8	0.393±0.00089	0.22759	8	0.4335±0.0169	3.900479
12	0.573667±0.00081	0.142329	12	0.636±0.00063	0.099443
16	0.732±0.00089	0.12219	16	0.813167±0.00098	0.120909
20	0.914667±0.00081	0.089267	20	1.019167±0.00075	0.073862

Table-3: Repeatability data of Leflunomide.

AT 286 nm			At 259 nm		
Con (µg/ml)	**Mean absorbance ±SD (n=6)**	**%RSD**	**Con (µg/ml)**	**Mean absorbance ±SD (n=6)**	**%RSD**
15	0.198 ± 0.00098	0.496	15	0.577 ± 0.00098	0.1706

Table-4: Repeatability data of Methotrexate.

AT 286 nm			At 259 nm		
Con (µg/ml)	**Mean absorbance ±SD (n=6)**	**%RSD**	**Con (µg/ml)**	**Mean absorbance ±SD (n=6)**	**%RSD**
12	0.574 ± 0.0012	0.2203	12	0.635 ± 0.0011	0.1840

Table-5: Intraday Precision data of Leflunomide.

AT 286 nm			At 259 nm		
Con (µg/ml)	**Mean absorbance ±SD (n=3)**	**%RSD**	**Con (µg/ml)**	**Mean absorbance ±SD (n=3)**	**%RSD**
10	0.144667±0.0015	1.055893	10	0.414667 ± 0.0020	0.502009
15	0.196667±0.0020	1.058474	15	0.577333 ±0.0015	0.264583
20	0.268±0.0026	0.987221	20	0.762 ±0.002	0.262467

Table-6: Intraday Precision data of Methotrexate.

AT 286 nm			At 259 nm		
Con (µg/ml)	**Mean absorbance ±SD (n=3)**	**%RSD**	**Con (µg/ml)**	**Mean absorbance ±SD (n=3)**	**%RSD**
8	0.396667±0.0025	0.63444	8	0.441667±0.0020	0.471321
12	0.575±0.002	0.347826	12	0.638±0.002	0.31348
16	0.735±0.002	0.272109	16	0.816333±0.0025	0.308282

Table-7: Interday Precision data of Leflunomide.

AT 286 nm			At 259 nm		
Con (µg/ml)	**Mean absorbance ±SD**	**%RSD**	**Con (µg/ml)**	**Mean absorbance ±SD**	**%RSD**
10	0.142667±0.0015	1.070695	10	0.420667±0.0051	1.219874
15	0.197333±0.0020	1.054898	15	0.577667±0.0055	0.953417
20	0.269667±0.0030	1.132899	20	0.766333±0.0056	0.742006

Table-8: Interday Precision data of Methotrexate.

AT 286 nm			At 259 nm		
Con (µg/ml)	**Mean absorbance ±SD**	**%RSD**	**Con (µg/ml)**	**Mean absorbance ±SD**	**%RSD**
8	0.399±0.005	1.253133	8	0.444667±0.0045	1.014074
12	0.575667±0.0050	0.874329	12	0.637667±0.0066	1.044171
16	0.741333±0.0080	1.081943	16	0.814±0.008	0.982801

Table 9: Accuracy data of Leflunomide and Methotrexate.

Name of sample	Level	Amount taken (µg/mL)	Amount added (µg/mL)	Recovered Concentration (µg/mL)	% Recovery ± SD (n=3)
LEF	80	10	8	17.86	99.24 ±0.19
	100	10	10	19.66	99.15 ±1.01
	120	10	12	21.90	99.56 ±1.10
MTX	80	2.5	2	4.61	102.4 ±0.18
	100	2.5	2.5	4.95	99.08 ±1.02
	120	2.5	3	5.50	100 ±1.3

Table 10: Assay Study Parameter.

Leflunomide			Methotrexate		
Concentration (µg/mL)	Amount found (µg/mL)	% Assay ± SD (n=3)	Concentration (µg/mL)	Amount found (µg/mL)	% Assay ± SD (n=3)
20	20.44	102.2 ± 0.744	5	4.92	98.4 ± 1.3

Conclusion

The proposed Spectrophotometric method was found to be simple, sensitive, accurate and precise for determination of LEF and MTX in synthetic mixture. The method utilizes easily available and cheap solvent for analysis of LEF and MTX hence, the method is economic for estimation of LEF and MTX in synthetic mixture. The common excipients and additives are present in the synthetic mixture form do not interfere in the analysis of LEF and MTX in method, Hence it can be conveniently adopted for routine quality control analysis of the drugs in mixture.

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