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Research Article

Development and Validation of UFLC Method for the Simultaneous Estimation of Pemetrexed and Folic Acid in API

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ABSTRACT

Cancer refers to a group of illnesses that result from cells in the body growing abnormally. These cells divide and produce new cells in an uncontrolled way that can spread throughout the body and can cause damage to essential organs. The main aim of my work is to perform the simultaneous estimation and method development of Pemetrexed and folic acid in combination by using UFLC with PDA detector. UFLC Chromatograph was performed with an (Shimadzu LC-20AD Pump Photo Diode Array Detector) analytical Kromasil C18 column using Phosphate buffer: Acetonitrile: Methanol (75:15:10) as the mobile phase. The flow rate was set at 1 mL/min and the injection volume was 20 µL. The minimally acceptable correlation coefficient (r^2) for the calibration curve was 0.99 and 0.992for Pemetrexed and folic acid respectively. Retention time of Pemetrexed and folic acid were 7.9 min and 3.6 min. respectively. The intra- and inter- day accuracies (% deviation) were within < ±10% for the QC samples

Keywords: UFLC, Retention time, Correlation coefficient, Pemetrexed, Folic acid.

1. INTRODUCTION

Cancer refers to a group of illnesses that result from cells in the body growing abnormally. These cells divide and produce new cells in an uncontrolled way that can spread throughout the body and cause damage to essential organs**.**When cancer spreads to other parts of the body this is called metastasis. Metastasis can occur when cancer cells enter the blood stream or lymph system. These systems circulate all over the body and allow the cells to travel. Tumors are masses (or lumps) that can develop as abnormal cells accumulate. Not all tumors are cancer. Benign (non-cancerous or nonmalignant) tumors do not spread to other parts of the body and are rarely life-threatening[1].

In 2004, Pemetrexed was approved by FDA for the treatment in malignant pleural mesothelioma, a type of tumor of the lining of the lung, in combination with cisplatin. In 2008, the FDA granted approval as a first-line treatment, in combination with cisplatin, against of locally-advanced and metastatic non-small cell lung cancer (NSCLC), in patients with non-squamous histology[2].

Pemetrexed (2*S*)-2-{[4-[2-2-amino-4-oxo-1, 7-dihydropyrrolo [2, 3-*d*] pyrimidin- 5yl (ethyl] benzoyl] amino} pentane dioicacidis afolate antimetabolite that exerts its effect by inhibiting multiple enzymes in the folate cascade. Pemetrexed primarily inhibits thymidylate synthase (TS), and to a lesser extent, glycin amide ribonucleotide formyl transferase (GARFT), dihydro folate reductase (DHFR) and amino imidazole carboxamide ribonucleotide formyl transferase (AICARFT) [3-7].

Vitamin B9, chemically known as either folic acid or pteroylglutamic acid,(2*S*)-2-[(4-{[(2-amino-4- hydroxypteridin-methyl] amino} phenyl) formamido] pentanedioic acid is one of the metabolically active compounds and is commonly referred

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to as folates. This water soluble vitamin helps the body to convert carbohydrates into glucose to produce energy[8].

The absence of folates in the human body can cause megaloblastic anemia, and affect mental and emotional health, along with other biological effects. As well as, the deficiency of this compound during pregnancy can increase the risk for neural tube birth defects including cleft palate, spinabifida (braindamage) [9, 10].

Pemetrexedas i.e, folate antimetabolite it diminishes the levels of folic acid in the body so Patients must take oral folic acid or a multivitamin containing folic acid (350 to 1000 micrograms) on a daily basis. At least five doses of folic acid must be taken during the seven days preceding the first dose of Pemetrexed and dosing must continue during the full course of therapy and for 21 days after the last dose of Pemetrexed. Patients must also receive an intramuscular injection of vitamin B12 (1000 micrograms) in the week preceding the first dose of Pemetrexed and once every three cycles thereafter. Subsequent vitamin B12 injections may be given on the same day as pemetrexed[11-13].

According to the literature survey it was found thatabove mentioned drugs were estimated either alone or combination with other drugs. As there are no currently manufacturing formulations containing both Pemetrexed and folic acid in combination in market so there is a need for the development of newer, simple, sensitive, accurate and economic analytical methods for effective estimation of Pemetrexed and folic acid in combinationinstead of taking folic acid alone after administration of Pemetrexed.

2. MATERIALS AND METHOD

2.1 UFLC method development

2.1.1. Chemicals and reagents

All solvents and reagents were of analytical and HPLC grade. Acetonitrile, Methanol, Water were purchased from Finar chemicals (Ahemadabad) and the drugs like Pemetrexed, Folic acid were obtained from Reddy's laboratories,(Hyderabad) and Loba chemicals (Bombay) respectively.

2.1.2. Instruments and chromatographic conditions

The Lab India UV-3000+ model were used to determine the absorption maxima (λ_{max}) of Pemetrexed and folic acid. UFLC Chromatograph (Shimadzu LC-20AD Pump Photo Diode Array Detector (Japan). The column and UFLC instrument was maintained at room temperature. The reverse phase chromatography was performed with an analytical Kromasil C18 column. Phosphate buffer: Acetonitrile: Methanol (75:15:10) was used as the mobile phase. The flow rate was set at 1 mL/min and the injection volume was 20 µL. The UFLC detector was set at a wavelength of 230nm[14, 15].

2.1.3. Preparation of standard solutions

Accurately weighed quantity of 10mg of Pemetrexed and folic acid weretransferred into 10 ml volumetric flasks separately, dissolved and diluted up to the mark with HPLC grade water to give a stock solution having a strength 1000µg/mL.

2.1.4. Standard graph procedure

Calibration curves were prepared by taking appropriate aliquots of standard Pemetrexed and folic acid stock solutions in different 10 ml volumetric flask and diluted up to the mark with mobile phase to obtain final concentrations of 5,10,15,20,25,30 µg/mL of Pemetrexed and 5, 10,15, 20,25, 30. µg/mL of folic acid. Standard solutions were injected through 20 µl loop system and chromatograms were obtained using 1.0 mL/min mobile phase flow rate. The effluent was monitored at 230 nm. Calibration curve was constructed by plotting average peak area against concentration and regression equations were computed.

2.2 HPLC method validation

HPLC method validation was carried out as per the procedure reported in literature[16-19].

2.2.1. Linearity

Calibration curves were plotted by taking Peak area of Pemetrexed and folic acid on Y-axis and Concentration of corresponding values on X-axis. The minimally acceptable correlation coefficient (r^2) for the calibration curve was 0.99 and 0.992 respectively.

2.2.2. Precision and Accuracy

In order to assess the intra-day, inter-day precision and accuracy, Pemetrexed and folic acid samples at (10µg/mL), (20µg/mL) and (30µg/mL) concentrations were prepared. The intra-day precision was assessed by calculating the coefficient of

variation (CV) for the analysis of samples in three replicates and twice in a day. And inter-day precision was determined by the analysis of samples on three consecutive days. Accuracy was calculated by comparing the measured values to the True values and was expressed in percent. The precision was accepted when the standard deviation for each concentration doesn't exceed ± 20, and accuracy was accepted when the average values are > 95% of true concentration except for the LOQ where the limit was > 92%.

2.2.3. Robustness

In the robustness study the influence of small deliberate variations of the analytical parameters on retention time of the drugs were examined. The following two factors were selected for change: flow rate of mobile phase (1, 0.9, and 1.1) and mobile phase P^H (2.5, 3) for Pemetrexed and folic acid.

2.2.4. Recovery

The percent recovery of Pemetrexed was calculated by measuring the peak area response of quality control samples at low, medium, high (10, 20 and 30 µg/mL) against the peak area response of unextracted quality control samples of equivalent concentrations.

2.2.5. Stability

The stability study of Pemetrexed (10, 20 and 30 µg/mL) was done under various conditions like 4 hr at room temperature, three freeze thaw cycles and storage at -80^{0}c for 1 month.

3. RESULTS AND DISCUSSION

3.1 .UV-Visible Spectrum of Pemetrexed and Folic acid

UV-Visible spectrum of Pemetrexed and folic acid was obtained in the wave length range of 200-400 nm by using Lab India UV-visible spectrometer (UV3000$^+$). Isobestic point of both drugs was found at a wave length of 230nm in water Fig-1 hence, wavelength of 230nm was used as λmax for the determination of Pemetrexed and folic acid using RP-HPLC.

3.2. HPLC method validation

Pemetrexed and folic acid was determined using a simple, sensitive and specific RP-HPLC method. An optimum separation was achieved using a composition of buffer: Acetonitrile : Methanol (75:15:10 % v/v) as a mobile phase.

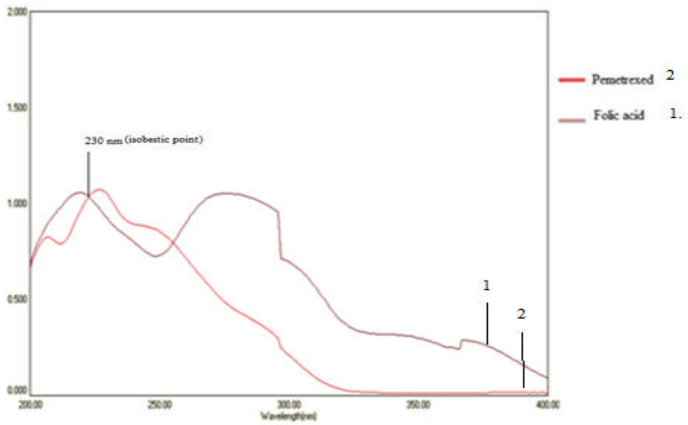


Figure-1: UV-Visible spectrum of Pemetrexed and folic acid within a range of 200-400nm

During the preliminary investigations different columns, different mobile phases were studied to select optimum conditions for the determination of Pemetrexed and folic acid, the separation of Pemetrexed and folic acid was achieved with a flow rate of 1mL/min at 230 nm. Under these conditions the retention times of Pemetrexed and folic acid were 7.9 min and 3.6 min. respectively, the chromatographic conditions were shown in Table-1 and Fig-2**.**

Table-1: Chromatographic conditions

HPLC System	Shimadzu
Column	Enable C_{18}
Flow rate	1mL/min
λ max	230nm
Mobile phase	Buffer: Acetonitrile: Methanol(75:15:10)
Retention time	Pemetrexed-7.9 Folic acid-3.6

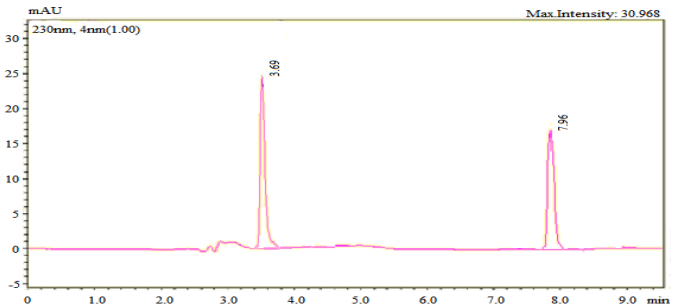


Figure 2: Sample Chromatogram of folic acid and Pemetrexed

3.2.1 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. this was shown in Fig-3.

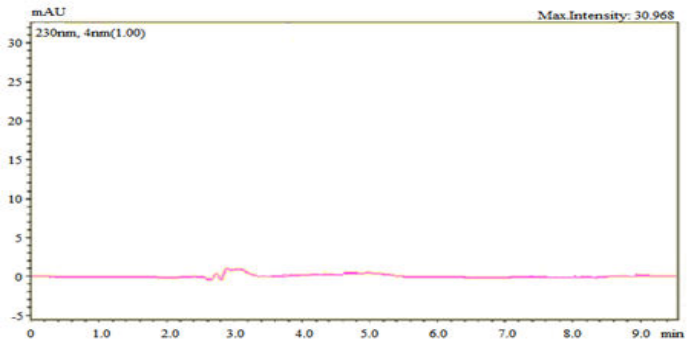


Figure 3: Blank Chromatogram

3.2.2 Linearity and range

The linearity of Pemetrexed and folic acid was found to be in the range of 5-30µg/mL with correlation co-efficient0.998 and 0.999 respectively .Calibration data were shown in Table-2 and3 and calibration curves were shown in Fig-4 and 5.

Table 2: Calibration data of Pemetrexed.

S. No	Concentration (µg/mL)	Peak area
1	5	108930
2	10	391508
3	15	787095
4	20	1259953
5	25	1804513
6	30	2280145

Table 3: Calibration data of folic acid

S. No	Concentration (µg/mL)	Peak area
1	5	168617
2	10	335342
3	15	562687
4	20	669430
5	25	836921
6	30	983699

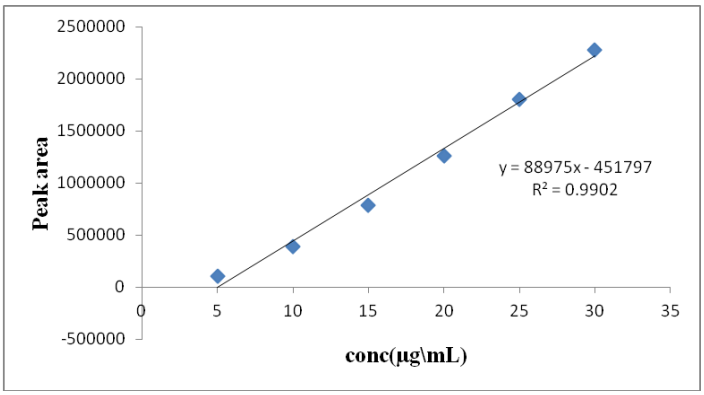
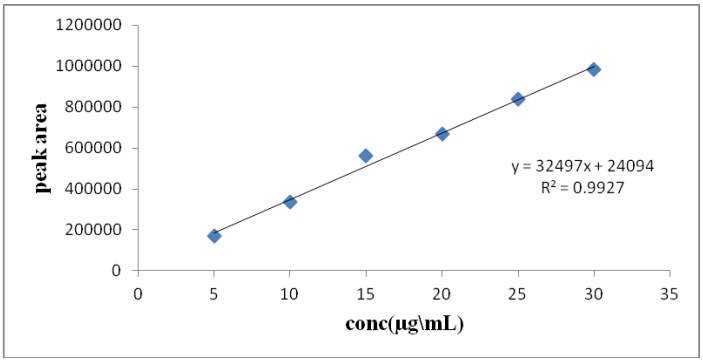


Figure 4: Standard graph of Pemetrexed



3.2.3 Precision and Accuracy

Intra-day and Inter-day accuracy and precision were determined using three quality control samples of concentrations of 10, 20 and 30 µg/mL and Intraday accuracy of Pemetrexed was found to be 99, 99.33 and 99.33% respectively. Inter- day accuracy of Pemetrexed was found to be 98, 98.33 and 99.44% respectively. Intraday accuracy of folic acid was found to be97.33, 99.5 and 99% respectively Therefore, the intra and inter- day accuracies (% deviation) were within < ±10% for the QC samples. The intra- and inter-day assay precision (CV) range of Pemetrexed from 1.53 to 0.33% and 2.10 to 0.19 % respectively. Intra- and inter-day assay precision (CV) range of folic acid from 1.18 to 0.67% and 1.02 to 0.19% these results indicated that the present assay has very good accuracy and precision, the results were shown in Table 4 and 5.

Table 4: Intra and Inter day variations of Pemetrexed

Intra day Concn (µg/mL)	**Trail-I**	**Trail-II**	**Trail-III**	**Mean**	**S.D**	**Accuracy % mean**	**%RSD**
10	9.9	9.8	10.1	9.93	0.152	99	1.537
20	19.9	20.1	19.6	19.86	0.251	99.33	1.266
30	29.8	29.7	29.9	29.8	0.57	99.33	0.335
Inter day Concn (µg/mL)							
10	10.1	9.8	10.1	9.8	0.208	98	2.109
20	19.3	19.9	19.8	19.66	0.321	98.33	1.634
30	29.9	29.8	29.8	29.83	0.057	99.44	0.193

Table 5: Intra and Inter day variations of Folic acid

Intra day Concn(µg/mL)	**Trail-I**	**Trail-II**	**Trail-III**	**Mean**	**S.D**	**Accuracy % mean**	**% RSD**
10	9.8	9.6	9.8	9.73	0.115	97.33	1.186
20	20.1	19.7	19.9	19.9	0.2	99.5	1.005
30	29.9	29.7	29.5	29.7	0.2	99	0.673
Interday Concn(µg/mL)							
10	9.8	9.9	9.7	9.8	0.1	98	1.020
20	19.9	20.1	19.9	19.9	0.115	99.83	0.578
30	29.8	29.8	29.83	29.8	0.057	99.44	0.193

3.2.4 Robustness

In the robustness study the influence of small deliberate variations of the analytical parameters on retention time of the drugs were examined. The following two factors were selected for change: flow rate of mobile phase (1, 0.9 and 1.1) and mobile phase P^H (2.5, 3) for Pemetrexed and folic acid. It was observed that there were no marked changes in the chromatogram, which demonstrated that the RP-HPLC method developed is robust the results were shown in Table 6 and 7.

Table 6: Robustness data of Pemetrexed

Parameter	Variations	Peak area mean	%RSD	Avg % RSD	Rt
Flow rate	1.0mL/min	136348	0.449907	0.382139	7.9
	0.9 mL/min	137200	0.323366		8.3
	1.1mL/min	135872	0.373144		7.4
P^H	2.5	136297	0.509555	0.423582	7.9
	3	136528	0.337609		7.7

Table 7: Robustness data of folic acid

Parameter	Variations	Peak area mean	%RSD	Avg % RSD	Rt
Flow rate	1.0mL/min	652295.7	0.101342	0.129341	3.6
	0.9mL:/min	652523	0.17772		3.9
	1.1mL/min	651821.3	0.10896		3.2
P^H	2.5	653189.3	0.205864	0.164908	3.6
	3	654882.3	0.123952		3.4

3.2.5 Limit of detection and Limit of quantification

Limit of detection (LOD) for Pemetrexed and folic acid was found to be 1.24µg/mL,0.69µg/mL respectively. Limit of quantification (LOQ) for Pemetrexed and folic acid was found to be 5.13µg/mL, 7.41µg/mL respectively.

3.2.6 Recovery

Percent recovery of Pemetrexed at low, medium, high (10, 20 and 30 µg/mL) was 92.82, 95.25 and 96.79% respectively; the results were shown in Table-8.

Table 8: Showing recovery of Pemetrexed

Intra-day	**Recovery (µg/mL)**				
	Replicate 1	**Replicate 2**	**Replicate 3**	**Mean**	**S.D**
10	94.8	85.2	98.48	92.827	6.856
20	95.28	98.56	91.92	95.253	3.320
30	97.184	95.378	97.832	96.798	1.272
Inter-day					
10	93.60	85.80	100.40	93.27	7.31
20	94.50	99.64	93.56	95.90	3.27
30	96.16	93.16	95.96	95.09	1.67

3.2.7 Stability

The stability study results of Pemetrexed under various conditions were summarized in Table-9.Pemetrexedat all QC (10, 20 and 30 µg/mL) levels was stable for 4 hour at ambient temperature, after three freeze thaw cycles, as well as after storage at -80^{0}c for 1 month. The high stable property of Pemetrexed suggested that no special care is needed during sample preparation.

Table 9: Stability data of Pemetrexed QC samples at various condition

Sample (µg/mL)	Condition	% Remaining ± SD
10	4 h at room temperature	98.9 ± 10.5
	3 freeze-thaw cycles	96.4 ± 10.9
	30 days storage at -80^{0}c	97.25 ± 7.1
20	4 h at room temperature	96.2 ± 5.2
	3 freeze-thaw cycles	98.6 ± 1.9
	30 days storage at -80^{0}c	100.58 ± 2.6
30	4 h at room temperature	99.3 ± 1.3
	3 freeze-thaw cycles	105.4 ± 3.1
	30 days storage at -80^{0}c	96.2 ± 4.1

4. CONCLUSION

A simple and isocratic reverse phase high performance liquid chromatography (RP-HPLC) method was developed and validated for quantitative estimation of Pemetrexed and folic acid in bulk drugs. The method was validated for accuracy, precision, linearity, limit of detection and limit of quantification. Pemetrexed and folic acid was analyzed by using Enable C_{18}G with isocratic elution of Buffer: Acetonitrile: Methanol as a mobile phase (75:15:10).The flow rate was set 1.0 mL/min and the analysis was performed at a wavelength of a 230 nm using photodiode Array (PDA) detector. The retention time for folic acid and Pemetrexed was respectively3.6mins and 7.9 mins. The proposed HPLC method is precise, accurate, specific and efficient and can be used in routine analysis in quality control laboratories.

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