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A Study of Method Development and Validation for Simultaneous Estimation of Pemetrexed and Cisplatin Using RP-HPLC

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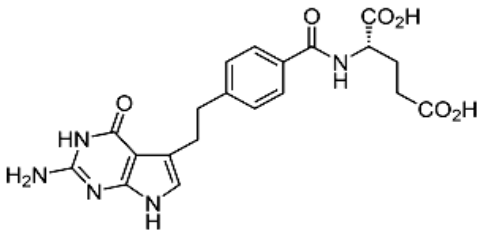
Abstract:

A novel, simple and accurate high performance liquid chromatographic method has been development with quantitative analysis of Pemetrexed and Cisplatin using Agilent eclipse XDB 150x4.6mm, 3.5µ column with a flow rate of 1ml/min. The buffer containing 1ml of ortho phosphoric acid dissolved in 1 lt of HPLC water, and the mixture of two components like Buffer and Acetonitrile in the ratio of 40:60 is used as mobile phase. The detection was carried out at 265nm. The proposed method shows good linearity in the concentration range from 50µg/ml to 750µg/ml for Pemetrexed and 1 µg/ml to 15 µg/ml of Cisplatin. Precision and recovery study results are in between 98-102%. In entire robustness conditions % RSD is below 2.0%. Degradation has minimum effect in stress condition and solutions are stable for 24hrs. Method validation is carried out according to ICH guidelines and the parameters are precision, accuracy, specificity, stability, robustness, linearity, limit of detection and limit of quantification are evaluated and the values are found to be within the acceptable limit.

Key words: ICH Guide lines, RP-HPLC, Pemetrexed, Cisplatin.

1. INTRODUCTION

Pemetrexed, sold under the brand name Alimta among others, is a chemotherapy medication for the treatment of pleural mesothelioma [1, 2] and non-small cell lung cancer [3]. Food and Drug Administration (FDA) approved pemetrexed for treatment of malignant pleural mesothelioma, a type of tumor of the mesothelium, the thin layer of tissue that covers many of the internal organs, in combination with cisplatin [4] for patients whose disease is either unresectable or who are not otherwise candidates for curative surgery. In September 2008, the FDA granted approval as a first-line treatment, in combination with cisplatin, against locally advanced and metastatic non-small cell lung cancer (NSCLC) in patients with non-squamous histology [5, 6].



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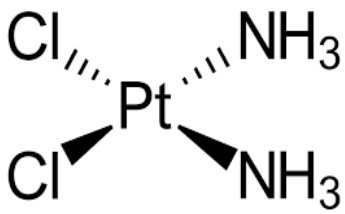

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Fig. No. 1: Chemical structure of (A) Pemetrexed and (B) Cisplatin

Figure 1 shows the chemical structures of Pemetrexed and Cisplatin.

Cisplatin is a chemotherapy [7, 8] medication used to treat a number of cancers. These include testicular cancer [9], ovarian cancer [10], cervical cancer [11], breast cancer [12], bladder cancer [13], head and neck cancer [14], esophageal cancer [15], lung cancer [16], mesothelioma [17], brain tumors [18] and neuroblastoma [19]. It is given by injection into a vein. Common side effects include bone marrow suppression [20], hearing problems, kidney problems, and vomiting [21]. Other serious side effects include numbness, trouble walking, allergic reactions, electrolyte problems [22], and heart disease [23]. Use during pregnancy can cause harm to the baby. Cisplatin is in the platinum-based antineoplastic [24] family of medications. It works in part by binding to DNA and inhibiting its replication.

2. MATERIALS AND METHODS

2.1 Materials: Acetonitrile, Ortho phosphoric acid, water were purchased from Merck (India) Ltd. Worli, Mumbai, India. All API's of Pemetrexed and Cisplatin as reference standards were procured from Glenmark pharmaceuticals, Mumbai.

2.2 Equipments: HPLC, make: Waters alliance e-2695 chromatographic system consisting of quaternary pump, PDA detector-2996 and chromatographic software Empower-2.0 was used.

2.3 Chromatographic Conditions: An instrument of HPLC system (Waters Alliance e2695 model) was used to develop the method and its validation. Empower 2.0 software was used to processing the data. The column was agilent eclipse XDB 150x4.6mm, 3.5µ dimensions. The selected drug was separated by using isocratic elution with a mobile phase of 0.1% ortho phosphoric acid buffer solution, acetonitrile in the ratio of 60:40. Flow rate of pump was set as 1.0ml/min. The UV detection was captured at 265nm. Injection volume fixed as 10µl and the diluent was same as the mobile phase.

Preparation of Mobile Phase:

Preparation of Buffer: 1ml of ortho phosphoric acid is dissolved in 1 lt of HPLC water and filter through 0.45 µ filter paper.

Preparation of Mobile Phase: Buffer: Acetonitrile (40:60)

Diluent: Mobile Phase is used as diluent.

2.4. Preparation of Standard solution: Weigh 500 mg of Pemetrexed and 10 mg of Cisplatin working standards into a 100ml volumetric flask, add 70ml of diluents sonicate for 15min to dissolve the contents, diluted volume with diluent. Further diluted 1ml to 10ml with diluents.

2.5 Wavelength optimization: The absorption spectra of solution of Pemetrexed and Cisplatin were scanned over the range of 200-400 nm by using PDA detector and the spectra was recorded. By observing the spectrum we can found that Pemetrexed and Cisplatin showing maximum absorbance at 265 nm. Hence, 265 nm was selected for method validation.

2.6 Method Validation

The analytical method was validated as per ICH Q2 (R1) guidelines for the parameters like system suitability, specificity, accuracy, precision, linearity, robustness, limit of detection (LOD), limit of quantification (LOQ), forced degradation and stability.

2.6.1 System Suitability

System suitability parameters were measured to verify the system performance. The parameters including USP plate count, USP tailing and % RSD are found to be within the limits.

2.6.2 Accuracy

Accuracy is the closeness of the test results obtained by the method to the true value. It was assessed by the recovery studies at three different concentration levels. In each level, a minimum of three injections were given and amount of the drug present, percentage recovery and related standard deviation were calculated.

2.6.3 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of other components (impurities, degradates or excipients), which may be expected to be present in the standard solution and standard solution. It was checked by examining the chromatograms of blank standard solutions and standard solutions spiked with Pemetrexed and Cisplatin.

2.6.4 Precision

Precision of an analytical method is the degree of agreement among individual test results. It was studied by analysis of multiple sampling of homogeneous standard solution. The precision of the present method was assessed in terms of repeatability, intra-day and inter day variations. It was checked by analyzing the standard solutions at different time intervals of the same day as well as on different days.

2.6.5 Linearity and range

Linearity of an analytical method is its ability to obtain results directly proportional to the concentration of the analyte in the standard solution within a definite range. The six series of standard solutions were selected for assessing linearity range. The calibration curve was plotted using peak area versus concentration of the standard solution and the regression equations were calculated. The least squares method was used to calculate the slope, intercept and correlation coefficient.

2.6.6 LOD and LOQ

LOD is the lowest amount of analyte in a standard solution that can be detected while LOQ is the lowest amount of analyte in a standard solution that can be determined with acceptable precision and accuracy. LOD and LOQ was separately determined based on the calibration curve. The LOD and LOQ for Pemetrexed and Cisplatin were determined by injecting progressively low concentrations of standard solutions using the developed RP-HPLC method. The LOD and LOQ were calculated as 3.3 s/n and 10s/n respectively as per ICH guidelines, where s/n indicates signal-to-noise ratio.

2.6.7 Stress degradation

Stress degradation should be no interference between the peaks obtained for the chromatogram of forced degradation preparations. Stress degradation studies were performed as per ICH guidelines Q_1A (R2). The degradation peaks should be well separated from each other and the resolution between the peaks should be at least 1.0 and the peak purity of the principle peaks shall pass. Forced degradation studies were performed by different types of stress conditions to obtain the degradation of about 20%.

2.6.8 Robustness

The robustness of an analytical procedure is a measure of its ability to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness study was performed by injecting standard solution into the HPLC system and altered chromatographic conditions such as flow rate (±0.2ml/min), organic content in the mobile phase (±10%). The separation factor, retention time and peak asymmetry were calculated by determining the effect of the modified parameters.

2.6.9 Stability

Analytical solution was prepared and injecting into the HPLC system at periodic intervals of 0 hours to 24 hours at 6hour intervals depending on the instrument utilization and sequence of injection.

3. RESULTS AND DISCUSSION

Optimization of Method and Standard solution concentration

For the first chromatographic conditions selected for method is reversed-phase HPLC with Agilent eclipse XDB 150x4.6mm, 3.5µ column with isocratic elution. Mobile phase is mixture of buffer and acetonitrile (40:60). The flow rate is 1.0ml/min and the column temperature is ambient.

The parameters of the developed and validated HPLC method are presented in table 1. Recovery data and peak sharpness depends to finalize the diluent and standard solution concentration and injection volumes were finalized greater threshold than the limit of quantification (LOQ). The isocratic was optimized to get the best

resolution. The optimized chromatographic conditions shown in table 1.

System suitability

The standard solution was introduced into HPLC system and found that system suitability parameters are within the limits. The percentage of RSD was calculated standard peak areas. The similar injections RSD percentage was observed and it is within the limit. The obtained results were presented in table 2 and the system suitability chromatogram was exhibited in the figure 2.

Table 1: Optimized HPLC method conditions

S. No.	Parameter	Method Conditions
1	Column	Agilent eclipse XDB 150x4.6mm, 3.5µ
2	Flow rate	1 ml/min
3	Wave length	265nm
4	Injection Volume	10µl
5	Run time	6 min
6	Mobile phase	0.1% OPA: ACN 40:60

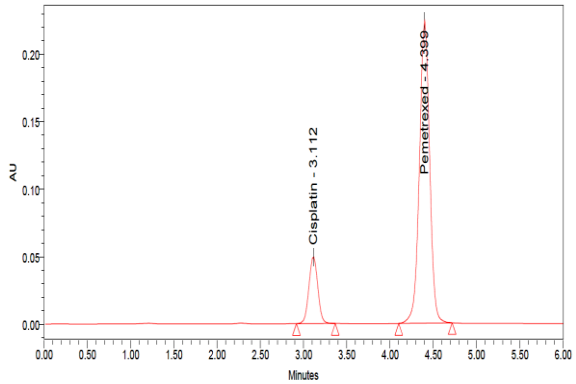


Fig. No. 2: Chromatogram of standard

Table 2: Results of system precision

S. No	System suitability parameter	Acceptance criteria	Drug Name	
			Pemetrexed	Cisplatin
1	% RSD	NMT 2.0	0.63	0.14
2	USP Tailing	NMT 2.0	1.02	1.24
3	USP Plate count	NLT 3000	3695	6531

Specificity

A study was conducted to establish the placebo interference. As per the test method, standard solutions are prepared equivalent weight of API and placebo with test concentration and then injected into HPLC system. Interference was not found for the chromatograms of placebo solution, empty cell solution at the retention time of Pemetrexed and Cisplatin.

The typical chromatogram of specificity was shown in the figures 3. Interference was not found for the chromatograms of placebo solution, blank solution at the retention time of Pemetrexed and Cisplatin.

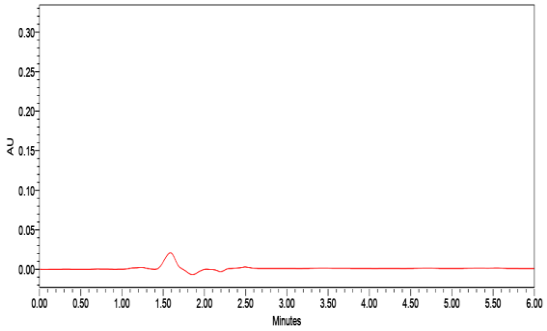


Fig. No. 3: Chromatogram of blank

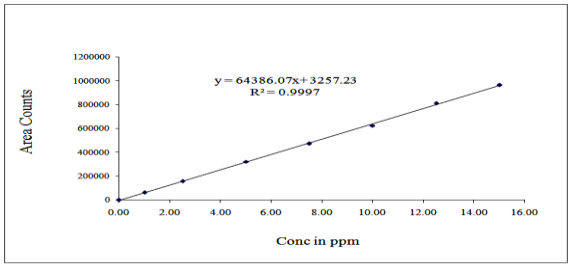
Linearity

Pemetrexed and Cisplatin linearity concentrations were prepared in the range of 50µg/ml to 750µg/ml of Pemetrexed and 1 µg/ml to 15 µg/ml of Cisplatin. The regression equations were found to be Y= 5001.85x+15389.99 (CC-0.9999) for Pemetrexed and Y=64386.07x+3257.23 (CC-0.9997) for Cisplatin.

The linearity plot was shown in figure 4 and the results were shown in table 3.

Table 3: Results of Linearity

S. No.	Pemetrexed		Cisplatin	
	Conc. (µg/ml)	Area	Conc. (µg/ml)	Area
Linearity-1	50.00	226954	1.00	62395
Linearity-2	125.00	617547	2.50	156935
Linearity-3	250.00	1202476	5.00	321578
Linearity-4	375.00	1857546	7.50	475102
Linearity-5	500.00	2506178	10.00	624711
Linearity-6	625.00	3120649	12.50	812574
Linearity-7	750.00	3725471	15.00	965302
Slope	5001.85		64386.07	
Intercept	15389.99		3257.23	
CC	0.9999		0.9997	



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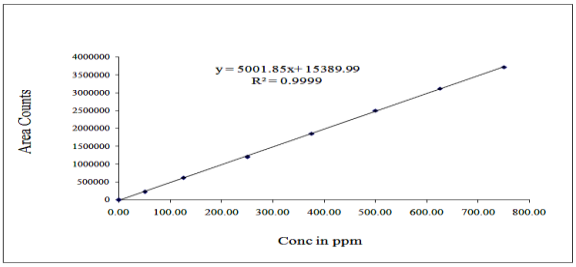

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Fig. No. 4: Linearity plot of (A) Pemetrexed and (B) Cisplatin

Robustness

In Robustness there is a small deviation in flow rate (±0.2ml) and organic solvent (±10%) in their chromatographic condition there is no significant change in RSD (%). The obtained results were presented in table 4.

Table 4: Results of Robustness

S.No	Parameter name	% RSD for purity	
		Pemetrexed	Cisplatin
1	Flow (0.8ml/min)	0.31	0.43
2	Flow (1.2ml/min)	0.69	1.25
3	Organic solvent (+10%) (33:67)	0.11	0.75
4	Organic solvent (-10%) (27:73)	0.84	1.16

Stability

Stability of Pemetrexed and Cisplatin were determined in standard solution was studying initial to 24hr at different time intervals at room temperature and at 2-8°C. There is no significant deviation of purity. The obtained results were listed in table 5.

Table 5: Results of stability

S.No	Stability	Purity of Pemetrexed in RT	Purity of Cisplatin in 2-8°C	Purity of Pemetrexed in RT	Purity of Cisplatin in 2-8°C
1	Initial	99.9	100	99.9	100
2	6Hr	99.6	99.7	99.5	99.8
3	12Hr	99.2	99.4	99.2	99.5
4	18Hr	98.8	99.1	99.0	99.2
5	24Hr	98.5	98.8	98.7	98.9

Precision

Precision of the method was established by injecting test preparation and tested through the complete analytical procedure from standard solution preparation to the final result. Repeatability assessed using a minimum of 6 determinations and calculated % relative standard deviation. The obtained results are tabulate in table 6.

Table 6: Results of Method precision

Analyte	Std Conc.	%RSD
Pemetrexed	500	0.53
Cisplatin	10	1.12

Intermediate Precision

Six replicates of a standard solution were analyzed on a different day, different analyst and different instrument. Peak areas were calculated which were used to calculate mean, % RSD values. The obtained results were presented in table 7.

Table 7: Results of Intermediate precision

Analyte	Std. Conc.	%RSD
Pemetrexed	500	0.27
Cisplatin	10	0.34

Limit of Detection and Quantification (LOD & LOQ)

LOD and LOQ were determined by calibration curve method. LOD and LOQ of the compound were determined by injecting progressively lower concentrations of standard solutions using developed RP-HPLC method. The slope method was used for estimation of LOD and LOQ and the equation used are LOQ= 10xσ/S and LOD= 3.3xσ/S, where S is the calibration curve slope and σ is the standard deviation of the response. The LOD and LOQ concentrations for Pemetrexed were 0.63µg/ml and 2.06µg/ml and for Cisplatin were 0.01 µg/ml and 0.04 µg/ml respectively. The typical chromatogram of LOD and LOQ were shown in figures 5 and 6.

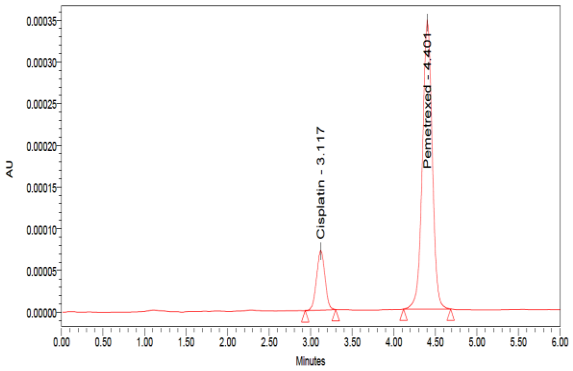

Fig. No. 5: Chromatogram of LOD

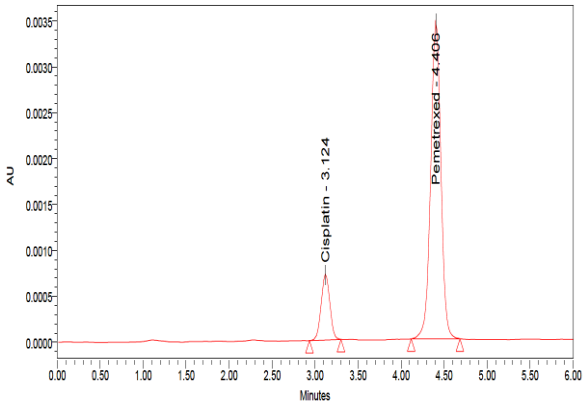

Fig. No. 6: Chromatogram of LOQ

Accuracy

Accuracy was determined by recovery studies which were carried out in three different concentration levels (50%, 100% and 150%). APIs with concentration of Pemetrexed 250, 500 and 750µg/ml and Cisplatin 5, 10, 15 µg/ml were prepared. The percentage recovery values were found to be in the range of 98-102%. Accuracy results were showed in table 8 and 9.

Degradation Effects

The Pemetrexed and Cisplatin standard stock was subjected into various forced degradation conditions to effect partial degradation of the drug. Forced degradation studies were performed to show the method is suitable for degraded products. Moreover, the studies provide information about the conditions in which the drug is unstable so that measures can be taken during formulation to avoid potential instabilities.

Table 8: Results of Accuracy of Pemetrexed

S. No.	% Level	% Recovery	Ave % Recovery
1	50	100.4	100.2
2		100.1	
3		100.1	
4	100	99.6	100.2
5		100.8	
6		100.3	
7	150	99.9	100.0
8		99.7	
9		100.5	

Table 9: Results of Accuracy of Cisplatin

S. No.	% Level	% Recovery	Ave %Recovery
1	50	100.5	100.4
2		100.2	
3		100.6	
4	100	100.6	100.4
5		100.5	
6		100.1	
7	150	100.7	100.6
8		100.3	
9		100.9	

Acid Degradation

In acid degradation procedure 5ml of standard solution transferred into a 50ml volumetric flask and add 1ml of 1N HCl heat for 30min at 60°C after that add 1ml of 1N NaOH then makeup to mark with diluent. Then the solution is filter through 0.45µ nylon syringe filter.

Alkali Degradation

The degradation procedure was performed as 5ml of standard solution transferred into a 50ml volumetric flask add 1ml of 1N NaOH heat for 30min at 60°C after that add 1ml of 1N HCl then make up to the mark with diluent. Then the solution is filter through 0.45µ nylon syringe filter.

Peroxide Degradation

The degradation procedure was performed as follow 5ml of standard solution transferred into a 50ml volumetric flask add 1ml of 30% H_2O_2 heat for 30min at 60°C then cool to makeup with diluent. Filter the solution with 0.45µ nylon syringe filter.

Reduction Degradation

The degradation procedure was performed as follows 5ml of standard solution transferred into a 50ml volumetric flask add 1ml of 30% sodium bicarbonate solution heat for 15min at 60°C then cool to makeup with diluent. Filter the solution with 0.45µ nylon syringe filter.

Thermal Degradation

1000mg of Pemetrexed and 50 mg of Cisplatin standard was exposed at 105°C for 3 hrs and the exposed standard solution was analyzed. 5mg of standard solution was transferred into 10ml volumetric flask. Add 5 ml diluent, sonicate to dissolve and diluted to volume with diluent. This solution is transferred into RB flask reflux at 60°C for 60mins. After that cool to room temperature. Further dilute 1ml to 10ml with diluents.

UV Degradation

In UV degradation procedure standard solution was exposed into sunlight for 12hr and reflux at 60°C for 30 min. The standard solution was injected into HPLC system.

Hydrolysis degradation

In hydrolysis degradation 5ml of standard solution transferred into a 50ml volumetric flask add 2ml of HPLC water and heat for 15min at 60°C then cool to makeup with diluent. Filter the solution with 0.45µ nylon syringe filter.

Forced degradation results were tabulated in table 10 and 11.

Table 10: Forced degradation results of Pemetrexed

Degradation Condition	% of Purity	% of Degradation	Purity Angle	Purity Threshold
Unstressed Degradation	99.9	-	0.562	10.247
Acid Degradation	84.79	15.21	0.547	10.265
Alkali Degradation	85.16	14.84	0.563	10.247
Peroxide Degradation	86.55	13.45	0.587	10.354
Reduction Degradation	87.44	12.56	0.521	10.228
Thermal Degradation	89.72	10.28	0.569	10.314
Photolytic Degradation	88.11	11.89	0.553	10.452

Table 11: Forced degradation results of Cisplatin

Degradation Condition	% of Purity	% of Degradation	Purity Angle	Purity Threshold
Unstressed Degradation	99.8	-	0.326	5.748
Acid Degradation	85.78	14.22	0.315	5.723
Alkali Degradation	85.31	14.69	0.347	5.758
Peroxide Degradation	84.46	15.54	0.359	5.744
Reduction Degradation	86.28	13.72	1.305	5.725
Thermal Degradation	88.99	11.01	1.342	5.749
Photolytic Degradation	87.54	12.46	1.336	5.783

CONCLUSION

A validated RP-HPLC method for stability indicating assay of Pemetrexed and Cisplatin was developed. The degradation behavior of the drug was investigated under (acid, base and neutral), oxidation, reduction, photolysis and thermal stress conditions. The drug was found to be stable in thermal, neutral conditions and unstable in remaining degradation conditions.

An isocratic RP-HPLC method for the determination of Pemetrexed and Cisplatin was developed and is precise and reliable. The regression line equation is capable of reliably predicting the drug concentration in the range of 50-750 µg/ml of Pemetrexed and 1-15 µg/ml of Cisplatin, from the peak area obtained. The method was successfully

validated and allowed the reliable, sensitive, robust and specific detection of Pemetrexed and Cisplatin.

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Conflicts Of Interest

The authors declare that there is no conflict of interests regarding to publication of paper.

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