Research Article

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR RELATED SUBSTANCE OF PEMETREXED DISODIUM

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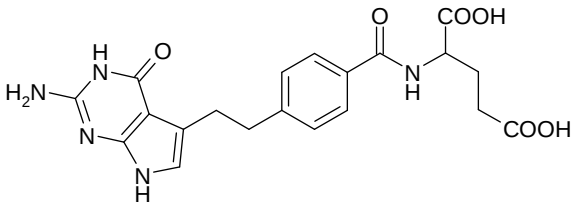
ABSTRACT

Simple and sensitive method for the quantification of related substance of pemetrexed disodium injection by RP-HPLC has been developed. Isocratic separation of pemetrexed disodium is carried out using a reversed-phase x-bridge column C18 (150 mm × 4.6 mm, 5 µm) with mobile phase consisting of acetonitrile and buffer (pH adjusted to 5 with orthophosphoric acid) in the ratio 15:85 (v/v) and quantified by UV detection at 230 nm. Analytical run time was 15 min. The assay exhibited good linear relationship with an accuracy and precision were over the concentration range of 0.1-10 µg/ml. This method can be used for routine analysis of related substances of pemetrexed disodium in injectables.

Keywords: HPLC, validation, related substances, pemetrexed disodium.

INTRODUCTION

Pemetrexed (figure 1) is an antifolate antimetabolite with multiple enzyme targets involved in both pyrimidine and purine synthesis.[1-3] It has shown encouraging activity in a wide range of tumors, including non-small cell lung carcinoma (NSCLC), malignant mesothelioma, and carcinomas of the breast, colorectum, uterine cervix, head and neck, and bladder.[4-7] It is marketed by Eli lilly and Company and Cadila Healthcare with the trade name Pemecad.



(2*S*)-2-{[4-[2-(2-amino-4-oxo-1,7-dihydropyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]- amino pentanedioic acid

Figure 1: Structure of Pemetrexed

Few studies on the estimation of pemetrexed content in pharmaceutical formulations, bulk drugs and biological fluids have been reported employing spectrophotometry, spectrodensitometry, voltametry, HPTLC and HPLC methods.[8-17] The proposed methods in the present study are economical and sensitive for the estimation of Pemetrexed Disodium and its related substances in bulk drugs and formulations.

MATERIALS AND METHODS

Finished product and placebo was collected from NATCO Pharma, Parenteral division, Nagarjunasagar, Hyderabad.

The composition of the formulation was presented below:

1. Pemetrexed Disodium 500 mg/vial

2. Mannitol 500 mg/vial

3. Water for Injection Qs to 10 ml

Methanol, Orthophosphoric acid, Acetonitrile, Potassium Dihydrogen phosphate, Sodium Dihydrogen phosphate were of HPLC grade purchased from Merck, Mumbai. Water for injection (0.22 µ filtered) and double distilled water for analytical purpose was obtained from milli-Q RO system.

The HPLC system consisted of a Alliance Water model liquid chromatographic pump, Rheodyne injection port (Rheodyne, Cotati, CA, USA) with a 20 µl sample loop and SPD-10A VP UV-Visible spectrophotometer detector. Data collection, integration and calibration were accomplished using Empower™ chromatography Data system.

HPLC Conditions

The chromatographic separation of Pemetrexed Disodium and its related substances were accomplished using x-bridge, 150x4.6mm, C18, 5 µm reverse phase analytical column. The mobile phase consisted of acetonitrile and buffer (pH adjusted to 5 with orthophosphoric acid) in the ratio 15:85 (v/v). Before use, the mobile phase was filtered by passing it through a 0.22 µm filter and the filtrate is degassed by using bath sonicator (Metler). The mobile phase was pumped at an isocratic flow of 1 ml/min at room temperature. The peaks were determined using a UV detector set at a wavelength of 230 nm. All the procedures were performed at ambient temperature.

Buffer Preparation

0.1% v/v of orthophosphoric acid in water for injection was prepared, mixed, degassed and filtered through 0.22 µ filter.

Preparation of mobile phase

85 Volumes of buffer and 15 volumes of acetonitrile were mixed and filtered through 0.22 µ porosity and degassed.

Standard Preparation

Pemetrexed disodium (20 mg) was weighed accurately and transferred to 100 ml volumetric flask, 25 ml water was added and make up to the mark with water. Further 1 ml of resulting solution was made up to 200 ml with water.

Sample preparation

The vial was reconstituted with 20 ml of water and transferred the contents into 250 ml volumetric flask. Each vial washed with 10 ml of water for 2-3 times and the final volume was made to 500 ml with water. Further 5 ml of above solution was diluted to 50 ml with water and filtered the solution.

Assay validation

The RP-HPLC assay validation was done as per ICH Q2A and Q2B guidelines which include system suitability, specificity, limit of detection (LOD) and limit of quantification (LOQ), determination of accuracy, precision, ruggedness, linearity and range.

System suitability

20 µl of Standard solution was injected and recorded the suitability parameters.

Specificity

A diluent was injected into the chromatographic system followed by the injection of excipient mixture (placebo), and standard in triplicate. A test solution with placebo and drug containing 0.5 % v/v of impurity was prepared and recorded the retention time respectively.

Accuracy

The samples were injected in triplicate by spiking test preparation with 80%, 100%, and 120% to the target concentration of 0.5 % v/v of impurity. The percentage recovery of impurities was calculated.

Precision

Precision is the measure of either the degree of reproducibility or of repeatability of the analytical method under normal operating conditions.

a) Method repeatability

The precision of test method was determined by spiking test preparation with impurities solution to get 0.5 % v/v of impurity in pemetrexed for injection 500 mg/vial (0.2 mg/ml of pemetrexed disodium). Repeatability was assessed using a minimum of six determinations and relative standard deviation was calculated.

b) Method reproducibility

Method reproducibility was demonstrated by six determinations of the same samples tested by another analytical group or in another laboratory (collaborative study). Similar procedure has been followed as that of method repeatability.

Ruggedness

The ruggedness of test method was determined by spiking test preparation with impurities to get 0.5 % v/v of impurity in pemetrexed for injection 500 mg/vial (0.2 mg/ml of pemetrexed disodium). Further it was tested through the complete analytical procedure from sample preparation.

Linearity and Range

Linearity and range was studied for the Pemetrexed Disodium and impurity in the range of LOQ levels to 120% (LOQ, 80%, 90%, 100%, 110%, & 120%) of the specified limit of each impurity (i.e. 0.5 % of Impurity). The degree of linearity was estimated by calculating the correlation coefficient, Regression coefficient (r^2), Y-intercept, residual sum of squares and Y-intercept / response at 100% of working concentration. Further a slope was drawn and reported by injecting each of the solutions (LOQ to 120%) in triplicate and 120% solution in six replicates. A plot of data was established for analyte response Vs Concentration.

RESULTS AND DISCUSSION

Chromatography

Sensitive, rapid, specific and reproducible RP-HPLC method has been developed and validated for quantitative determination of related substances of Pemetrexed Disodium. The representative chromatograms of Pemetrexed Disodium standard, Pemetrexed Disodium sample and blank were shown Fig. 2, 3 and 4. The retention time of Pemetrexed Disodium was 8.2 min and the peaks were sharp. A good baseline separation of Pemetrexed Disodium was also observed.

System suitability

The results were shown in table 1. System suitability parameters were in compliance with the guideliness.

Table 1: System suitability parameter results

Parameter Description	Acceptance Criteria	Results
The % RSD of peak area response	Not more than 10.0	0.70
The tailing for pemetrexed peak	Not more than 2.0	1.16
The plate count for pemetrexed peak	Not less than 7500	15621

Specificity

The results were shown in table 2. No interference was observed from placebo and diluent and it meets acceptance criteria. Hence the test method is specific and selective for estimation of relative substance in pemetrexed injection 500 mg/vial.

Table 2: Specificity parameter results

Sample details	Retention times obtained
Diluent-1	Nil
Diluent-2	Nil
Diluent-3	Nil
Placebo-1	Nil
Placebo-2	Nil
Placebo-3	Nil
Standard-1	8.124
Standard-2	8.122
Standard-3	8.121
Sample-1	3.2,3.4,4.1,4.3,6.4,8.1,17.3,24.45
Sample-2	3.2,3.4,4.1,4.3,6.4,8.1,17.3,24.45
Sample-3	3.2,3.4,4.1,4.3,6.4,8.1,17.3,24.45

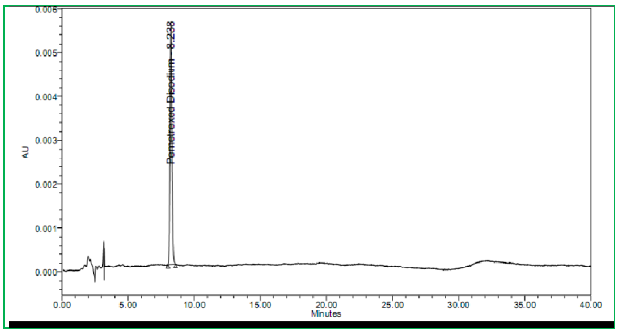


Figure 2: Standard Chromatogram

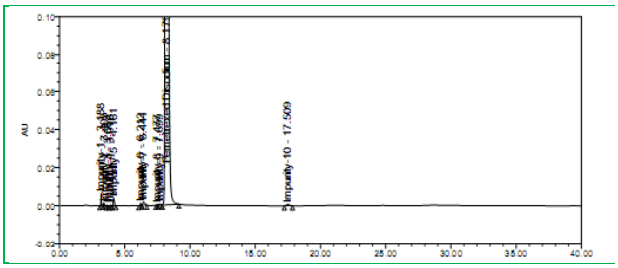


Figure 3: Sample Chromatogram

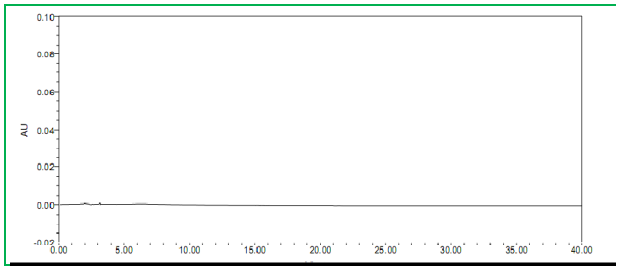


Figure 4: Blank Chromatogram

Limit of detection (LOD)

S/N ratio of pemetrexed peak is 3.90 and the limit of detection for pemetrexed was declared as 0.025 ppm concentration. The limit of detection (LOD) for ethanol was found to be 0.025 ppm. The result has been shown in table 3 and 4.

Table 3: Limit of Detection

Concentration	Area response of pemetrexed disodium	S/N Ratio
0.0025 ppm	884	3.90
Observation	Detected at 0.025	3.90

Table 4: Solvent analysis

Product conc.	Area response of pemetrexed
Pemetrexed: 0.025 ppm	884
	967
	1030
	1024
	1079
Acceptance criteria	S/N ratio shall be 2:1or 3:1

Limit of quantification (LOQ)

%RSD for six replicate injections of pemetrexed at LOQ level was found to be 0.085 ppm. For precision, the %RSD for six replicate injections of pemetrexed at about limit of quantification are within the limit. Thus the limit of quantification for ethanol is declared as 0.085 ppm. Concentration and S/N ratio is 11.89. %RSD for six replicate injections of pemetrexed at LOQ level are within the limit. The result has been shown in table 5.

Table 5: Limit of Quantification

S. No	Area response of pemetrexed
1	4032
2	4344
3	4190
4	4221
5	4512
6	4450
Average	4292
%RSD	4.15
Acceptance criteria	The %RSD shall not be more than 10.0

Accuracy

The results were shown in table 6. It was found that the accuracy (recovery) for the average of triplicate from each concentration levels were within 85% to 115% (i.e. 104.19% for 80% level, 100.43% for 100% level and 100.38% for 120% level).

Table 6: Accuracy

Sample No →	MIX-1 (80%)	MIX-II (100%)	MIX-II (120%)
	Areas obtained		
Preparation 1	45166	54483	65225
Preparation 2	45065	54258	65003
Preparation 3	44852	54018	64990
Average	45028	54253	65073
Obtained assay mg per ml	0.00075	0.0009	0.00108
Spicked assay mg per ml	0.00072	0.0009	0.0011
% Recovery	104.19	100.43	100.38
Parameter description	Acceptance criteria		
The % Recovery	Should be between 85 and 115		

Precision

Precision is the measure of either the degree of reproducibility or of repeatability of the analytical method under normal operating conditions.

Method repeatability

The %RSD of six sample preparations of pemetrexed Injection 500 mg /vial was 8.583.

It was observed that, %RSD of the six replicate samples from sample preparations within the limit. Hence the

method is precise for estimation of relative substances in pemetrexed Injection 500 mg /vial. The results were shown in table 7.

Table 7: % RSD of Related Substance obtained

S. No.	% RSD of Impurity
1.0	0.267
2.0	0.239
3.0	0.287
4.0	0.280
5.0	0.242
6.0	0.236
Average area obtained	0.259
SD	0.022
%RSD	8.583

Method reproducibility

The %RSD of six sample preparations of pemetrexed Injection 500 mg /vial was 8.203.

It was found that, the relative standard deviation of the six replicate preparations of both analysts, were less than 10.0%. And the RSD between the assay results obtained by both analysts found less than 3.0%. Hence the test method is found precise and reproducible for pemetrexed Injection 500 mg /vial. The results were shown in table 7.

Ruggedness

The %RSD of six sample preparations of pemetrexed injection 500 mg /vial was 0.31.

The %RSD of between the average assay results obtained by both analysts was found 1.94%. The relative standard deviation of the six replicate preparations of both analysts on different days, were less than 10.0%. And the RSD between the average RS results obtained by both analysts found less than 10.0%. Hence the test method is found precise and rugged for pemetrexed Injection 500 mg /vial. The relative standard deviation of the six replicate preparations on different equipment, were less than 10.0 % and the %RSD between the average assay results obtained by both equipments found less than 10.0%. Hence the test method is found precise and rugged for pemetrexed Injection 500 mg /vial and it meets the acceptance criteria. The results were shown in table 8 and 9.

Table 8: % RSD of Related Substance obtained

S. No.	% RSD of Impurity
1.0	0.274
2.0	0.233
3.0	0.268
4.0	0.285
5.0	0.246
6.0	0.240
Average area obtained	0.258
SD	0.021
%RSD	8.203

Table 9: Comparative results of precision and ruggedness study of pemetrexed disodium for Injection 500 mg /vial

Sample No	% Impurity	
	Day-1 /Analyst-1 (Data from Precision study)	Analyst-2
T1	0.267	0.187
T2	0.239	0.196
T3	0.287	0.191
T4	0.280	0.190
T5	0.242	0.184
T6	0.236	0.189
Average	0.259	0.190
SD	0.022	0.0015
%RSD	8.66	2.10

Linearity and Range

It was found that, Correlation coefficient and y-intercept at 100% values are within the limits from range 80% to 120% of target concentration. Hence the test method is found linear for the estimation pemetrexed impurity in pemetrexed injection 500 mg/vial. Further it met the acceptance criteria. The results were shown in table 10 and a graph was plotted for RS linearity and shown in figure 5.

Table 10: Linearity and Range

Sample No	% LA	
	Equipment-QA/163	Equipment-QA/117
T1	0.267	0.180
T2	0.239	0.180
T3	0.287	0.181
T4	0.280	0.182
T5	0.242	0.184
T6	0.236	0.180
Average	0.259	0.181
SD	0.022	0.0017
%RSD	8.66	0.96

Table 11: Linearity

Conc (w.r.t. test)	Prep	Conc taken (%w.r.t)	Average (Conc)	Area Response	Corrected Area
80	1	0.734	0.734	44349	44347
	2	0.734		44617	
	3	0.734		44074	
90	1	0.826	0.826	49678	49457
	2	0.826		49479	
	3	0.826		49214	
100	1	0.918	0.918	55144	54920
	2	0.918		54900	
	3	0.918		54717	
110	1	1.010	1.010	60215	60332
	2	1.010		60399	
	3	1.010		60381	
120	1	1.101	1.101	65738	65743
	2	1.101		65900	
	3	1.101		65590	
Acceptance criteria	Correlation coefficient				1.00
	Report the Y-intercept and slope				58473
	% Y-intercept should be within + 3.0				1293.00
	Graph should be visually linear				2.354

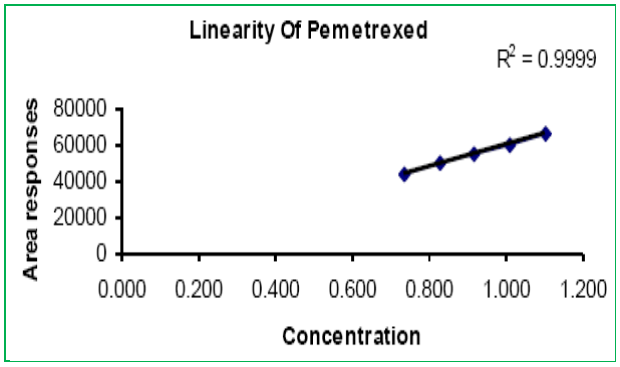


Figure 5: Linearity plot

CONCLUSION

A high-performance liquid chromatographic method for the determination of pemetrexed impurity in pemetrexed injection has been developed and validated. It has been shown to be accurate, precise and sensitive. This method can be used for analysis of related substances of pemetrexed disodium. Several studies in the literature for the determination of the tested compound depends on UV, HPTLC or volumetric methods, few used RP-HPLC. Nevertheless, the results of the study indicate that the developed HPLC method is simple, precise, accurate and less time consuming.

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