

Validated reverse phase HPLC method for the determination of pemetrexed disodium in pharmaceutical dosage forms

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ABSTRACT

A simple and isocratic reverse phase high performance liquid chromatography (RP-HPLC) method was developed and validated for quantitative determination of Pemetrexed Disodium in bulk drug samples and formulations. The method was validated for system suitability, accuracy, precision, linearity, specificity, solution stability, limit of detection and limit of quantitation. Pemetrexed Disodium was analyzed by using Zorbax SB – C18 (250 mm x 4.6 mm, 5 µm) at ambient temperature, with isocratic elution of Buffer:Acetonitrile (90:10). The flow rate was set at 0.9 ml/min and the analysis was performed at a wavelength of 265 nm using Photo Diode Array (PDA) detector. Efficient UV detection at 265 nm enabled determination of Pemetrexed disodium without any interference from injectable solution excipients or solvents. The retention time (RT) for Pemetrexed disodium was around 6 min. The calibration curves were linear over a concentration range from 0.06248 mg to 0.49984 mg/ml. Limit of detection (LOD) for Pemetrexed disodium was 0.0002522 mg/ml and Limit of quantitation (LOQ) was 0.0001261 mg/ml. The developed method was successfully applied to estimate the amount of Pemetrexed disodium in formulations.

Key words: Pemetrexed disodium, high performance liquid chromatography, reverse phase liquid chromatography, validation.

INTRODUCTION

Pemetrexed is an anticancer drug indicated in the treatment of pleural mesothelioma as well as non small cell lung cancer. Its chemical name is 2-[4-[2-(4-amino-2-oxo-3, 5, 7-triazabicyclo [4.3.0] nona-3, 8, 10-trien-9-yl) ethyl] benzoyl] aminopentanedioic acid. Its chemical structure is shown in figure 1. It is a folate analog metabolic inhibitor that exerts its action by disrupting folate-dependent metabolic processes essential for cell replication. In vitro studies have shown that pemetrexed inhibits thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycylamide

ribonucleotide formyltransferase (GARFT), which are folate-dependent enzymes involved in the *de novo* biosynthesis of thymidine and purine nucleotides.

Few HPLC methods for quantitative determination of Pemetrexed disodium in formulations were reported in the literature and these reports mainly included the determination of Pemetrexed disodium and its metabolites in biological fluids¹⁻⁵. The aim of the work is to develop and validate a rapid, economical and sensitive HPLC method for quantitative determination of Pemetrexed disodium in bulk drug samples and

injectable preparations. In order to minimize batch-to-batch variation there is an immense need for developing a rapid, sensitive and validated analytical method for day-to-day analysis of the drug in pharmaceutical dosage forms.

MATERIAL AND METHODS

Chemicals and reagents

Pemetrexed disodium bulk drug (99.80 % purity) and formulations were kind gifts from TherDose Pharma Pvt. Ltd., Hyderabad, India. Acetonitrile (HPLC grade) was obtained from Rankem, India.

Instrumentation

The HPLC system consisted of a Shimadzu LC-2010 AHT module with PDA Detector. Data acquisition was performed by LC solutions software operated on a Pentium® IV microprocessor. Zorbax SB – C18 (250 mm x 4.6 mm 5 µm) at ambient temperature, with isocratic elution of Buffer: Acetonitrile (90:10). The flow rate was set 0.9 ml/min and the analysis was performed at a wavelength of 265 nm using Photo Diode Array (PDA) detector. The mobile phase was degassed and filtered through 0.2 µm membrane filter before pumping into HPLC system.

Preparation of solutions

Buffer

About 13.8 gms of disodium hydrogen phosphate dihydrate in 1000 ml of milli Q water and adjust the pH to 6.5 with orthophosphoric acid.

Preparation of drug stock solution

The stock solution of Pemetrexed disodium was prepared by dissolving accurately weighed quantity of 62.48 mg of the drug in 50 ml of milli Q water (concentration 1.2496 mg/ml).

Method validation

System suitability

The system suitability was assessed by replicate analysis of six injections of the drug at a concentration of 0.251 mg/ml. The acceptance criteria were not more than 2% for the percentage relative standard deviation (% RSD) for the peak area and 1.5% for retention time of Pemetrexed disodium peaks. The number of theoretical plates

should not be less than 2500.

Determination of Limit of Detection and Limit of Quantitation (Sensitivity)

The limit of detection is determined by calculating the signal to noise ratio and by comparing test results from samples with known concentrations of analyte with those of blank samples and establishing the minimum level at which the analyte can be reliably detected⁶⁻¹⁰. The LOD and LOQ of the method were demonstrated by preparing 4 level concentrations of 0.01%, 0.03%, 0.05% and 0.07% of the target concentration of 0.25 mg/ml. About 20 µl of Pemetrexed disodium level solutions were injected into the chromatographic system and observed S/N ratio.

Calculation

$$\text{Height} / \text{baseline noise} \times 1000$$

Linearity (Calibration curve)

Standard Stock solution

Accurately weighed 62.48 mg of Pemetrexed disodium drug substance and transferred to a 50 ml volumetric flask. Dissolved and brought to volume with the milliQ water and mixed. (Concentration 1.2496 mg/ml)

Level solutions

A series of solutions in 7 concentrations of 25%, 50%, 75%, 100%, 125%, 150% and 200% were prepared using the standard stock solution.

The linearity of the method was demonstrated by above prepared level concentration 50%, 75%, 100%, 125%, 150%, 150%, 200% of the target concentration of 1.2496 mg/ml. About 20 µl of each of the above prepared solutions was injected onto the chromatographic system connected to ZORBAX SB-C18 column (in duplicate) and calculated the average area in each case.

Accuracy and precision

Accuracy was calculated with respect to above prepared solution at the levels of 75%, 100% and 125% of the normal or target concentration. The accuracy of the method was demonstrated through recovery experiment on 3 samples at concentration 75.08%, 99.83% and 124.95% of the

actual concentration employed in the usual procedure. The actual concentration employed in the determination was 0.25 mg/ml of Pemetrexed disodium containing the excipients used in the inventor formulation and the recovery was calculated in each of the case using the regression line equation.

Demonstration of precision was done under two categories. The injection reproducibility was assessed by injecting six replicate injections of the standard solution of Pemetrexed disodium and the relative standard deviation of the replicate injections was calculated.

Injection Reproducibility

Relative standard deviation of RT and Area of the above system suitability solution were calculated for injection reproducibility.

Method Precision

Six individual preparations of Pemetrexed disodium drug substance were prepared with target concentration of about 0.25 mg/ml for Method Precision.

Procedure

Injected 50 µL of above preparations onto the chromatographic system connected to a Zorbax SB-C18 column in duplicate and recorded the chromatograms. Calculated the peak areas due to pemetrexed peak.

Application of the Method to Dosage Forms

Statistical Analysis

Calculated the Mean, Standard Deviation and the Relative Standard Deviation for the assay of 6 preparations.

Calculations

Calculated the content of the Pemetrexed disodium in the sample solution using the following formula:

$$\frac{A_T}{A_S} \times \frac{W_S}{W_t} \times P$$

Where,

A_T = Average area of the sample preparation

A_S = Average area of the standard preparation

P = Potency of the standard

W_T = Concentration (mg/ml) of the sample solution

W_S = Concentration (mg/ml) of the standard solution

Solution stability

The validity of the assay solutions was demonstrated for a period of 48 hours by chromatographing the same solution at periodic intervals.

Procedure

About 20 ml of the standard solution was injected at 0, 24 and 48 hrs onto the column of the chromatographic system and recorded the peak areas in the chromatograms.

Calculations

Observed the areas of the peaks of Pemetrexed disodium injected at the mentioned time intervals and calculated their corresponding concentrations.

Specificity

The specificity of the method was demonstrated by interference check by injecting the diluent blank and placebo solution to determine whether any peaks in the diluent and placebo solution are co-eluting with Pemetrexed disodium peaks.

RESULTS AND DISCUSSION

Method development and optimization

Pemetrexed is freely soluble in water. The drug can be separated on a Zorbax SB – C₁₈ column in reverse phase mode. The optimization of the method development was done by isocratic elution. The following isocratic program was used for method development.

Mobile Phase: Buffer: Acetonitrile (90:10)

The peak shape and symmetry were good with above isocratic elution and Pemetrexed disodium peaks were resolved with greater than 1.0 resolution at a flow rate of 0.9 ml/min.

Method Validation

System Suitability

Resolution was not less than 1, number of theoretical plates was not less than 2500, and

percentage relative standard deviation (%RSD) for Peak Area was not more than 2.0 % for Pemetrexed disodium peaks.

The %RSD of peak area for the drug is within 2% indicating the suitability of the system (Table 1). The efficiency of the column as expressed by number of theoretical plates for the 6 replicate injections was 4038 and the tailing factor was 1.2.

Determination of Limit of Detection and Limit of Quantitation (Sensitivity)

Limit of detection (LOD) for Pemetrexed disodium was found to be 0.00002522mg/ml and

Limit of quantitation (LOQ) Pemetrexed disodium was 0.0001261mg/ml.

Linearity

The calibration curve constructed was evaluated by its correlation coefficient. The peak area of the drug was linear in the range of 0.06248 to 0.49984 mg/ml.

Statistical Evaluation

A graph between the concentration and the average area was plotted. Points for linearity were observed. Using method of least squares a line of best fit was taken and calculated the Correlation

Table 1: System suitability study of pemetrexed disodium

Injection Number	Retention Time	Peak Area	Theoretical plates	Tailing factor
Injection – 1	6.39	8800832	3909	1.2
Injection – 2	6.38	8800684	3969	1.2
Injection – 3	6.36	8795368	4027	1.2
Injection – 4	6.36	8797957	4084	1.2
Injection – 5	6.36	8795009	4113	1.3
Injection – 6	6.35	8797044	4124	1.3
Mean	6.37	8797816	4038	1.2
RSD	0.23	0.03	2.12	0.85

Table 2: Linearity of pemetrexed disodium

S. No	Linear Solution Range	Concentration (mg/ml)	Peak Area	Mean Area
1	25%	0.06248	2189693 2187002	2188348
2	50%	0.12496	4358186 4365030	4361608
3	75%	0.18744	6470401 6471647	6471024
4	100%	0.24992	8567376 8574663	8571020
5	125%	0.3124	10688729 10726874	10707802
6	150%	0.37488	12785867 12772837	12779352
7	200%	0.49984	16400194 16403657	16401926

Table 3: % Recovery of pemetrexed disodium

Level	Amount added Pemetrexed disodium (conc. mg/ml)	Mean Area response	Amount found (Pemetrexed disodium (conc. mg/ml)	% of recovery
75%	0.18744	6474303	0.18766	100.1
	0.18744	6470401	0.18754	100.1
	0.18744	6471647	0.18758	100.1
100%	0.24992	8565240	0.24950	99.8
	0.24992	8567376	0.24956	99.9
	0.24992	8574663	0.24978	99.9
125%	0.3124	10675880	0.31192	99.8
	0.3124	10688729	0.31230	100.0
	0.3124	10726874	0.31343	100.3
			Mean	100.0
			SD	0.16
			% of RSD	0.16

Calibration curve equation values:

Slope : 33811172
 Intercept : 129369
 R² value : 0.999

Table 4: Method precision of pemetrexed disodium

S.No	Solution ID	Conc. (mg/ml)	Peak Area	Mean Area	Assay
1	Standard solution	0.2532	8645995	8638178	89.0
			8641508		
			8634963		
			8634646		
			8635867		
			8636089		
2	Preparation – 1	0.2450	8379781	8309856	88.5
			8239931		
3	Preparation – 2	0.2554	8715431	8716292	89.0
			8717153		
4	Preparation – 3	0.2556	8695489	8692563	88.7
			8689637		
5	Preparation – 4	0.2554	8713372	8715005	89.0
			8716637		
6	Preparation – 5	0.2426	8228981	8226842	88.5
			8224703		
7	Preparation – 6	0.2560	8693832	8691851	88.6
			8689870		
Mean	88.7				
SD	0.26				
% of RSD	0.29				

Coefficient, Slope, and Y-intercept. Average areas were shown in table 2 and the plot was shown in figure 2.

These experiments indicated that there was a linear relationship between the amounts of analyte and the areas within the range studied. The chromatograms of Pemetrexed disodium extracted from the formulation and pure Pemetrexed disodium can be observed in Figures 3 and 4.

Accuracy

Accuracy of the method was determined by recovery experiments. Accuracy was calculated with respect to above prepared solution at the levels of 75%, 100% and 125% of the normal or target concentration. The accuracy of the method was demonstrated through recovery experiment on 3 samples at concentration 75.08%, 99.83% and 124.95% of the actual concentration employed in the usual procedure. The actual concentration employed in the determination was 0.25 mg/ml of Pemetrexed disodium. The amount of Pemetrexed disodium found in each of these test solutions was calculated using the calibration curve. The table 3 summarizes the amount added vs. amount found and calculated using the calibration curve and percentage recovery. The results revealed that there was a strong correlation between the amount added and amount found for Pemetrexed disodium.

Precision

The precision of the method was demonstrated through two parameters which are

injection reproducibility and the method precision.

For method precision, 6 individual samples were prepared from the same batch of the drug substance and measured the individual peak retention time and peak area. The relative standard deviation was calculated for these six samples. For injection reproducibility, six injections from the same standard preparations were made and the relative standard deviation for the duplicate injections was calculated. The results for method precision were given in table 4.

Application of the method to dosage forms

The HPLC method developed is sensitive and specific for the quantitative determination of Pemetrexed disodium. The method is validated for different parameters and hence has been applied for the estimation of drug in pharmaceutical dosage forms. Injections of inventor formulation from TherDose Pharma Pvt. Ltd, India, were evaluated for the amount of Pemetrexed disodium present in the formulation. Each sample was analyzed in triplicate and the amount of Pemetrexed disodium in the formulation was 99.86% (Table 5). None of the injection excipients interfered with the analyte peak as seen in the figures 3 and 4.

Solution stability

The validity of the assay solutions was demonstrated for a period of 48 hours by chromatographing the same solution at periodic intervals.

Table 5: Assay content of pemetrexed disodium

S.No	Solution ID	Conc. (mg/ml)	Mean Area	Assay
1.	Standard solution	0.2532	8638178	89.0
2.	Sample solution	0.2510	8558735	88.88

Table 6: Solution stability data

Time period	Peak Area	Assay Percent	% of initial
Initial	8688061	89.5	100.0
24 hrs	8635998	89.5	100.0
48 hrs	8607730	89.2	99.7

Procedure

About 20 μ l of the standard solution was injected at 0, 24 and 48 hrs onto the column of the chromatographic system and recorded the peak areas in the chromatograms.

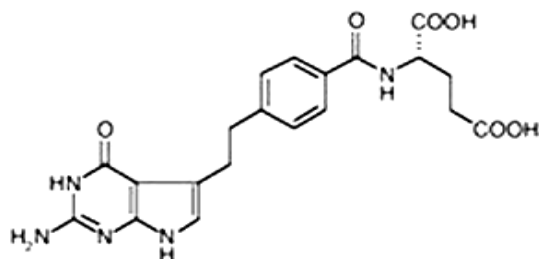


Fig. 1: Chemical structure of pemetrexed

Calculations

Observed the areas of the peaks of Pemetrexed disodium injected at the mentioned time intervals and calculated their corresponding concentrations (Table 6).

Specificity

The specificity of the method was demonstrated by checking the interference of any other peaks with drug peaks. This was performed by injecting the diluent blank and placebo solution to determine whether any impurity peaks in the diluent and placebo solution peaks are co-eluting with Pemetrexed disodium peaks. No interference of peaks eluted in the blank and placebo solution with Pemetrexed disodium peaks was observed.

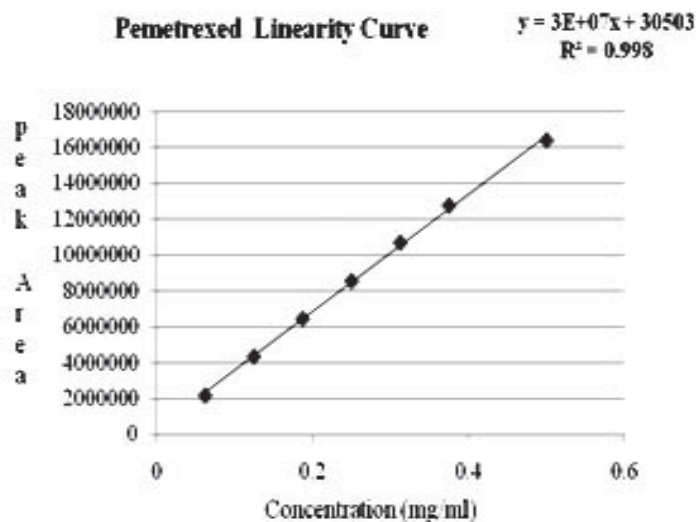


Fig. 2: Linearity for pemetrexed disodium

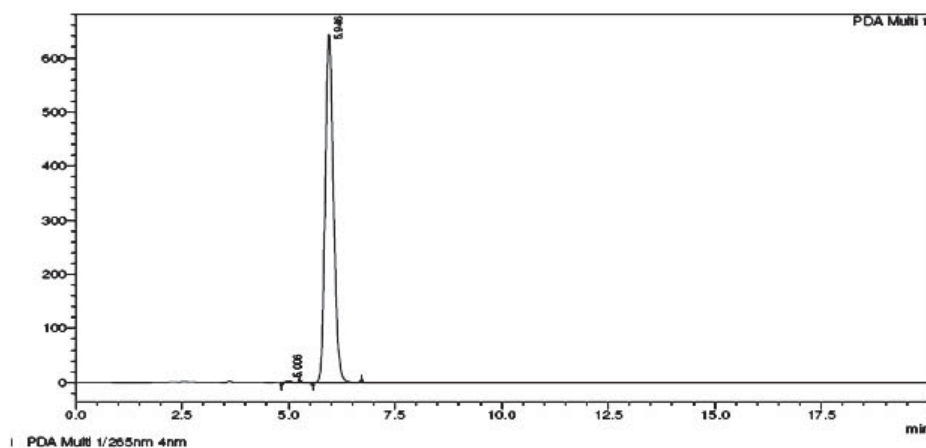


Fig. 3: QC sample chromatogram of Pemetrexed Disodium extracted from formulation

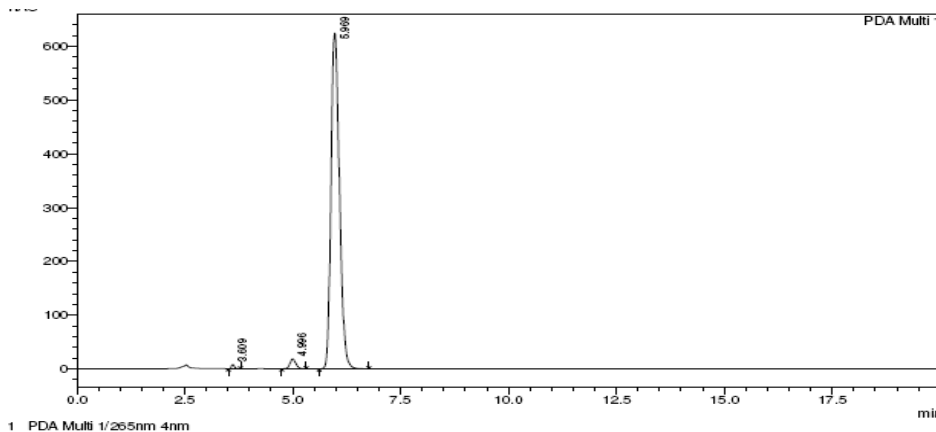


Fig. 4: Chromatogram of Pemetrexed Disodium extracted from standard solution

CONCLUSION

A simple and isocratic reverse phase high performance liquid chromatography (RP-HPLC) method was developed and validated for quantitative determination of Pemetrexed disodium in bulk drug samples and formulations. The method was validated for accuracy, precision, linearity, specificity limit of detection and limit of quantitation. Pemetrexed disodium was analyzed by using Zorbax SB – C₁₈ (250 mm x 4.6 mm, 5 μm) at ambient temperature, with isocratic elution of Buffer:Acetonitrile as a mobile phase (90:10). The flow rate was set 0.9 ml/min and the analysis was performed at a wavelength of 265 nm using Photo Diode Array (PDA) detector. Efficient UV detection at 265 nm enabled determination of Pemetrexed disodium without any interference from injectable solution excipients or solvents. The retention time (RT) for Pemetrexed disodium was around 6.00 min.

The calibration curves were linear over a concentration range from 0.06248 mg/ml to 0.4998 mg/ml. Limit of detection (LOD) for Pemetrexed disodium was 0.00002522 mg/ml and Limit of quantitation (LOQ) Pemetrexed disodium was 0.0001261 mg/ml. The developed method was successfully applied to estimate the amount of Pemetrexed disodium in formulations. The proposed HPLC method is precise, sensitive, accurate, specific and efficient and can be used in routine analysis in quality control laboratories.

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