DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF PEMETREXED DISODIUM, ITS IMPURITIES AND DEGRADATION STUDIES OF BULK DRUG

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Abstract: Pemetrexed disodium is a novel, multi-targeted antifolate drug used for wide variety of solid tumors. Three process related impurities formed in novel synthetic route of Pemetrexed disodium bulk drug synthesis. The impurities were characterized by using NMR, MS/MS, FT-IR and UV spectroscopic techniques. Method validation is carried on Inertsil ODS C-18-3V (250x4.6mm, 5µ particle size) column with mobile phase methanol, water and o-phosphoric acid (0.5%) 50:45:5 (v/v/v) flow rate of 0.8 mL/minute at 220 nm wavelength (λmax). The developed method is validated with respect to system suitability, specificity, accuracy, precision, linearity, LOD and LOQ. The LOD and LOQ for the Pemetrexed disodium and its each of three process related impurities were found to be 0.2µg/mL and 0.6µg/mL respectively. The bulk drug was subjected to oxidative, acidic, basic, photolytic and thermal stress conditions at zero and 48 hours. The maximum degradation of the drug was shown 38% for thermal stress.

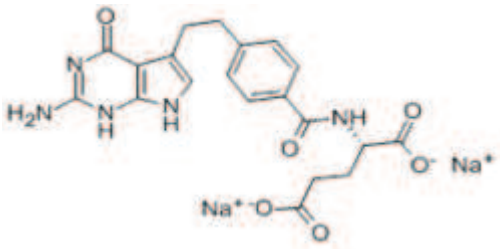
Keywords: Characterization, Forced Degradation, HPLC, Impurities A, B and C, Linearity; Pemetrexed Disodium and Validation.

1. Introduction: Pemetrexed disodium is a novel, multi-targeted antifolate drug that has demonstrated promising clinical activity in a wide variety of solid tumors [1]. As a single agent, Pemetrexed disodium shows good activity against non-small cell lung cancer, squamous-cell carcinoma of head and neck, colon cancer and breast cancer. It appears to be particularly active in combination with Cisplatin against non-small cell lung cancer and mesothelioma [2]. Pemetrexed disodium is currently used in phase III studies for mesothelioma and non-small cell lung cancer (NSCLC) [3]. It has shown good activity in preclinical models with human tumor cells and xenografts [4-7]. The stability of Pemetrexed disodium solution for intravenous administration has been extensively studied [8]. Olga Michalak et al [9] have developed

synthesis and physicochemical characterization of the impurities of Pemetrexed Disodium, an anticancer drug. Warner et al [10] have reported developed purity control strategy for Pemetrixed disodium and validation of associated analytical methodology. Rakesh Gupta K et al [11] have reported development and validation of RP-HPLC method for related substance of Pemetrexed disodium.

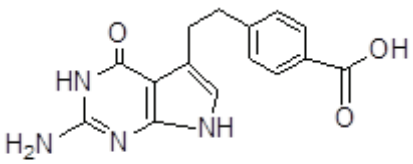
Suresh Kumar A and Devendra Singh R [12] have reported development and validation of Pemetrexed by RP-HPLC method in bulk drug and pharmaceutical dosage forms. Hemchand S et al [13] have developed enantiomeric separation and validation of D-isomer in Pemetrexed disodium an anti-cancer agent using chiral HPLC. Hemchand S et al [14] have developed a new validated stability-indicating gradient RP-HPLC method for the determination of Pemetrexed disodium and its process related substances.

However, to the best of our knowledge there are no analytical methods available for simultaneous determination of three process related impurities formed in novel synthetic route of Pemetrexed disodium bulk drug. It is felt necessary to develop a stability-indicating liquid chromatographic method for the quantitative determination of Pemetrexed disodium and its process related impurities. The current research work also deals with the forced degradation of the bulk drug substance under stress conditions like acid hydrolysis, base hydrolysis, oxidation, heat and light.

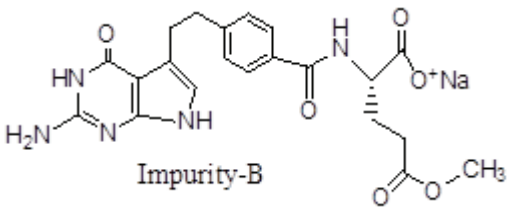


Pemetrexed disodium

L-Glutamic acid, N-[4-[2-(2-amino-4,7-dihydro-4-oxo-1H-pyrrolo[2,3-d]pyrimidin-5-yl) ethyl]benzoyl] disodium salt or 2S-2-{[4-[2-(2-amino-4-oxo-1,7-dihydropyrrolo [2,3-*d*] pyrimidin-5-yl)ethyl]benzoyl] amino}pentane dioic acid disodium salt.



Impurity-A



Impurity-B

4 [2 - (2 – amino - 4, 7 – dihydro – 4 – oxo -1H - pyrrolo [2,3-d] pyrimidinyl-5yl) ethyl] benzoic acid	N - [4 - [2- (2 amino - 4,7 – dihydro – 4 – oxo- 1H – pyrrolo [2,3d] pyrimidine - 5yl)ethyl] benzoyl] – L - glutamic acid 5 methyl ester

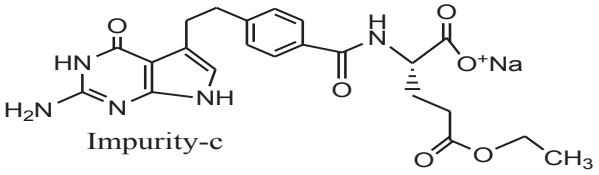
 Impurity-c	N - [4 - [2 - (2 amino - 4, 7, dihydro - 4 - oxo - 1H - pyrrolo [2,3d] pyrimidine -5-yl)ethyl] benzoyl] - L-glutamic acid - 5 ethyl ester

Fig. 1: Structures of Pemetrexed Disodium and Its Impurities

2. Experimental

2.1. Apparatus/Instruments: The HPLC from Shimadzu, Kyoto, Japan was used. The system was composed of two LC-10AT VP pumps, a SPD-M10AVP diode array detector, a SIL-10AD VP auto injector, a DGU-12A degasser and SCL-10A VP system controller. The chromatographic and the integrated data were recorded using HP-Vectra (Hewlett packard, Waldbronn, Germany) computer system. Elico, model LI 120; pH meter was used. Characterization of drug and impurities were carried on UV, FT-IR, ^{1}H NMR and MS/MS.

2.2. Materials and Reagents: All the reagents were of analytical reagent grade unless stated otherwise. Glass-distilled and deionized water (Nanopure, Barnstead, USA), HPLC-grade methanol, sodium hydroxide, hydrochloric acid, hydrogen peroxide and ortho-phosphoric acid (S.D.Fine chem, Mumbai, India) were used. Samples of Pemetrexed disodium (reference standard) and its process related substances were a kind of gift from M/s Bio Leo analytical labs, India Pvt. Ltd, Hyderabad.

2.3. Optimized Chromatographic Conditions:

Mobile phase : Methanol, water and o-phosphoric acid (0.5%) 50:45:5 (v/v/v)
Column : Inertsil ODS C-18-3V (250x4.6mm, 5µ particle size)
Flow rate : 0.8 mL/min
Injection volume : 20 µL
Detector : Photo diode array (PDA)
Wavelength (λmax) : 220 nm
Column temperature : Ambient
Diluent : Mobile phase
Run time : 16 minutes
pH : 6

2.4. Preparation of Mobile Phase: Methanol, water and o-phosphoric acid (0.5%) were taken in the ratio of 50:45:5 (v/v/v). The resultant solution was thoroughly mixed and filtered through a (PTFE) filter of 0.45 µm pore size using vacuum pump and degassed by sonication to expel the dissolved gases in solvent system.

2.5. Preparation of Standard Solutions: Standards of Pemetrexed disodium and its three process related impurities (10 mg each) were accurately weighed, transferred into 100 mL volumetric flasks, dissolved in mobile phase as diluents and made up to the mark with the

mobile phase to get 100 µg/mL each of Pemetrexed disodium and its process related impurities in solution.

2.6. Preparation of Working Standard Solution: 7mL of the above standard solution for each impurity is transferred into 100mL volumetric flask and made up to the mark with mobile phase to get the concentration of 70µg/mL.

2.7. Spectral Reports: The chemical characterization of Pemetrexed disodium and its impurities A, B and C were performed by spectroscopic data shown in the Figure 2 (a) to (h).

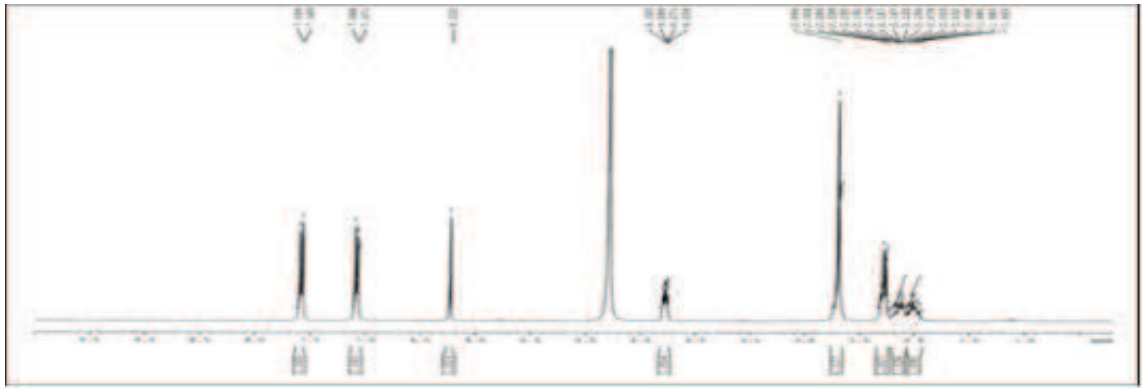


Fig. 2(a): Proton NMR Spectral Report of Pemetrexed Disodium

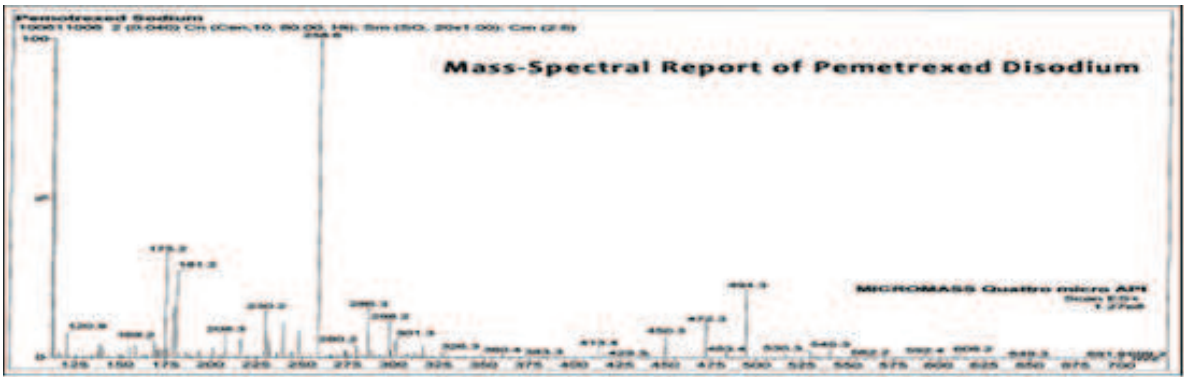


Fig. 2(b): Mass Spectral Report of Pemetrexed Disodium

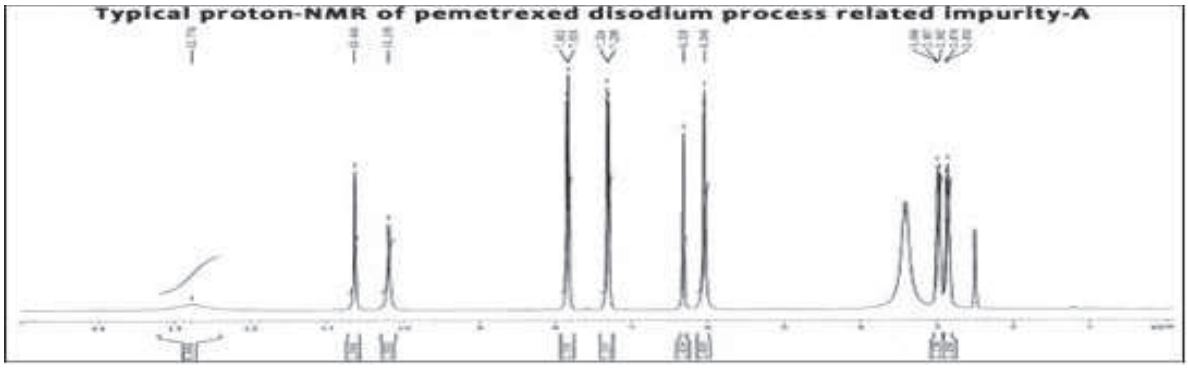


Fig. 2(c): Proton NMR Spectral Report of Impurity-A

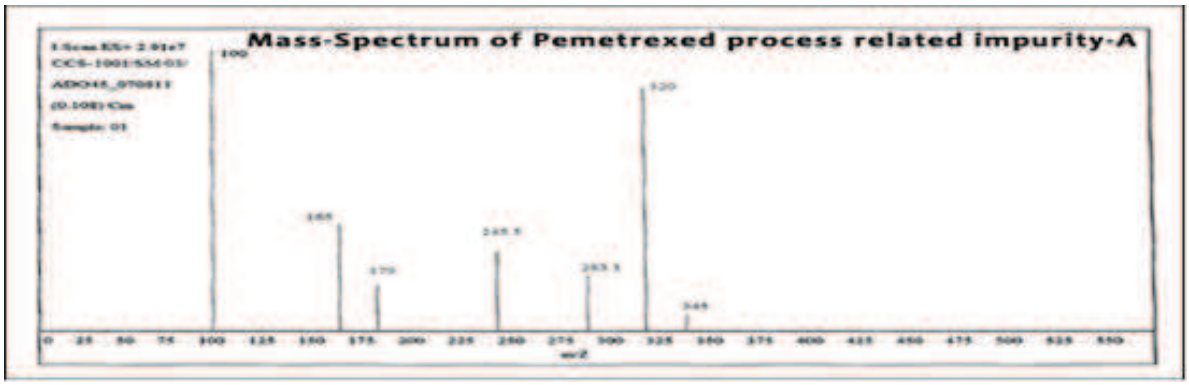


Fig. 2(d): Mass Spectral Report of Impurity-A

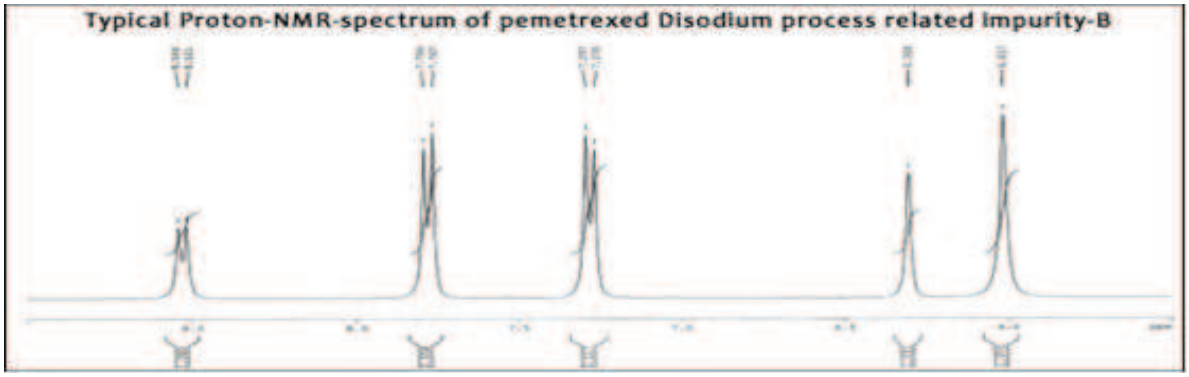


Fig. 2(e): Proton NMR Spectral Report of Impurity-B

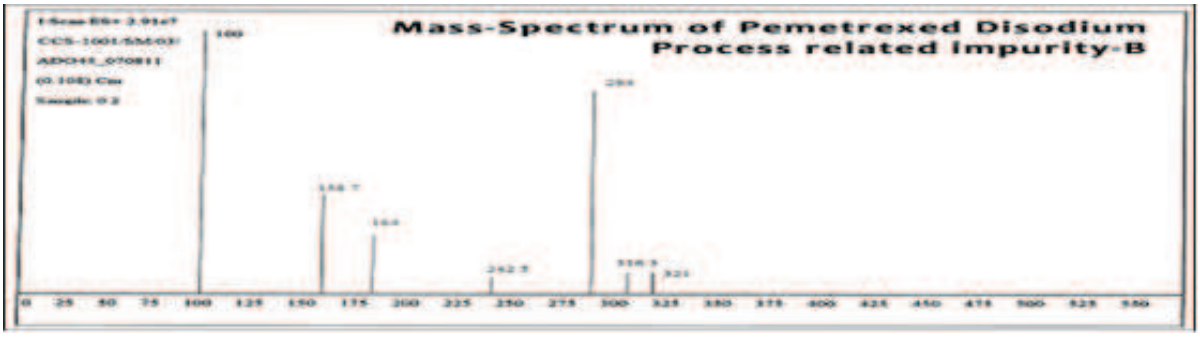


Fig. 2(f): Mass Spectral Report of Impurity-B

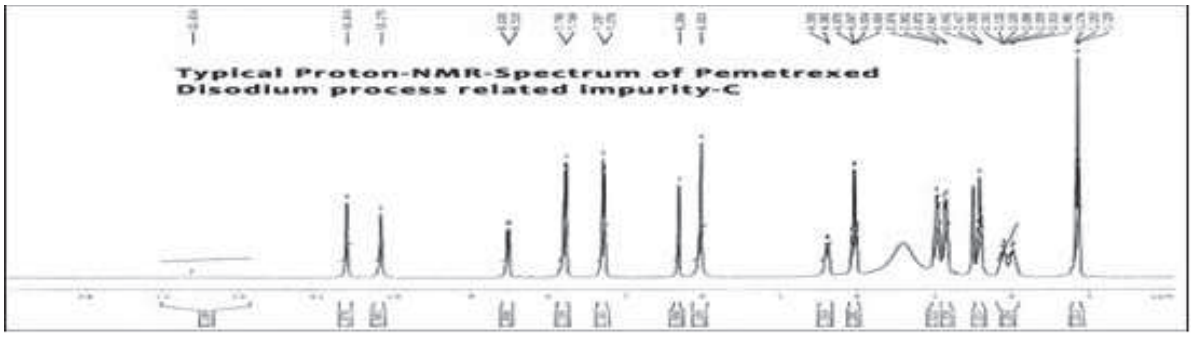


Fig. 2(g): Proton NMR Spectral Report of Impurity-C

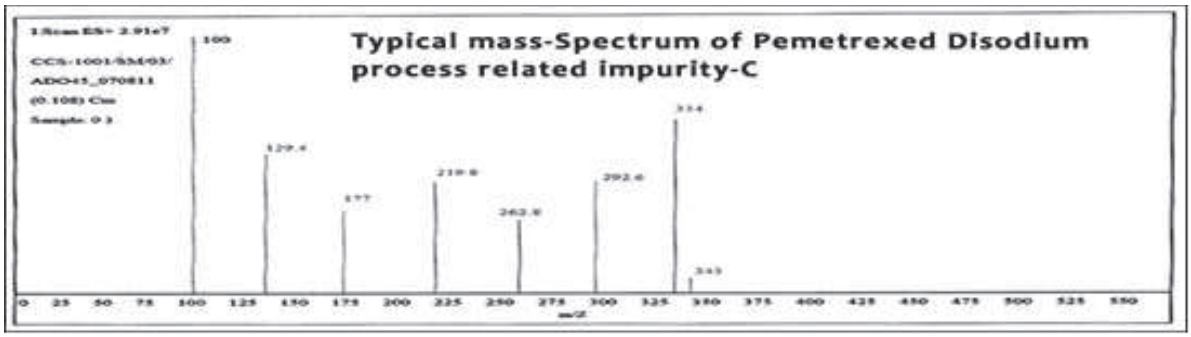


Fig. 2(h): Mass Spectral Report of Impurity-C

3. Results and Discussions:

Method Validation: The developed method is validated with respect to system suitability, specificity, accuracy, precision, linearity, LOD and LOQ.

3.1. System Suitability: The system suitability was conducted using 70 µg/mL of all impurities spiked to Pemetrexed Disodium at 100µg/mL and evaluated by making three replicate injections. Pemetrexed disodium and its process related impurities system suitability data is given in Table I.

Table.1 System Suitability Data of Pemetrexed Along with its Impurities

Sample	Retention time in minutes	Peak area	Resolution	Theoretical plates	Tailing factor
Pemetrexed disodium	3.43	99558	-------	8524	1.04
Impurity A	8.69	156265	5.05	25452	1.66
Impurity B	7.65	335282	22.78	25743	1.02
Impurity C	12.70	103567	20.95	100350	0.96

3.2. Specificity: For specificity determination the three impurities are spiked to Pemetrexed disodium and the response of each analyte in the mixture was compared with that of individual solutions. Solutions of blank and standard solution of Pemetrexed disodium were evaluated along with impurity solutions. The specificity chromatograms & data of the Pemetrexed disodium and impurities are shown in Figures 3 and Table 2.

Table 2: Specificity Study of Pemetrexed Disodium along with its Impurities

Sample	Retention in time (min)	Retention time in spiked solutions (min)
Pemetrexed disodium	3.45	3.67
Impurity-A	8.47	8.60
Impurity-B	7.51	7.66

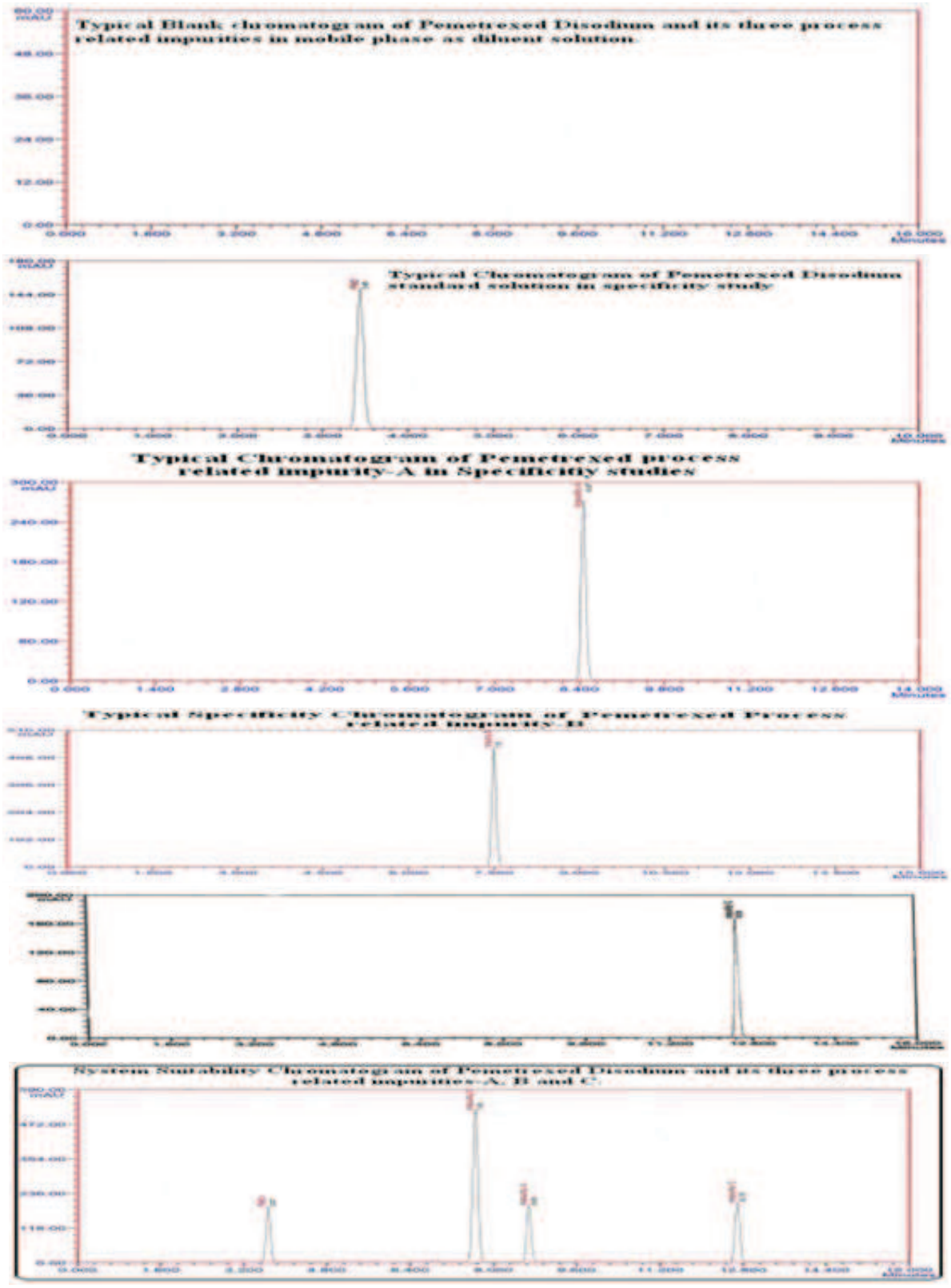


Fig. 3: Specificity Chromatograms Of Pemetrexed Disodium Along With Its Impurities

3.3. Precision: The system and method precision were conducted using 70 µg/mL of three impurities spiked to Pemetrexed disodium (100µg/mL) and evaluated by making six replicate injections. System and method precision data were kept in the Table 3 and 4 respectively.

Table 3: System Precision Values of Pemetrexed Disodium and its Impurities

Injection No	Pemetrexed peak area	Impurity B peak area	Impurity A peak area	Impurity C peak area
1	96004	347923	164770	103601
2	95432	340770	161377	100214
3	95623	348661	164308	102957
4	96430	348917	162997	100630
5	96216	345186	161394	103860
6	95734	346422	160572	100530
Mean	95906.5	346313.16	162569.66	101965.33
Std.Dev.	378.05489	3062.44604	1722.85817	1682.79739
%RSD	0.39	1.12	1.06	1.65

Table.4: Method Precision Values of Pemetrexed Disodium and its Impurities

Injection No	Pemetrexed peak area	Impurity B Peak area	Impurity A Peak area	Impurity C Peak area
1	99558	335282	156159	103567
2	98807	337307	163037	100058
3	97452	334283	160279	101404
4	96490	337729	159502	98076
5	99085	332696	160888	101600
6	98892	332744	161883	99548
Mean	98380.66	335006.83	160291.33	100708.8
Std.Dev.	1162.96	2179.95	2371.81	1905.22
%RSD	1.18	0.65	1.48	1.89

3.4. Linearity: The linearity of Pemetrexed disodium impurities are studied by preparing standard solutions at five different levels ranging from 40µg/mL to 80µg/mL. The data were subjected to statistical analysis using a linear-regression model, the regression equations and coefficients (r2) are given in Table 5 and calibration graphs are shown in Figure 4 and linearity chromatograms are shown in Figure 5.

3.5. Range: The standard aliquots for pemetrexed disodium and its three impurities were prepared within the concentrations of the range at 40, 50, 60, 70 and 80 µg/mL. From the results obtained in linearity studies with reference to concentration of analytes, the range was found to be 40µg/mL to 80µg/mL.

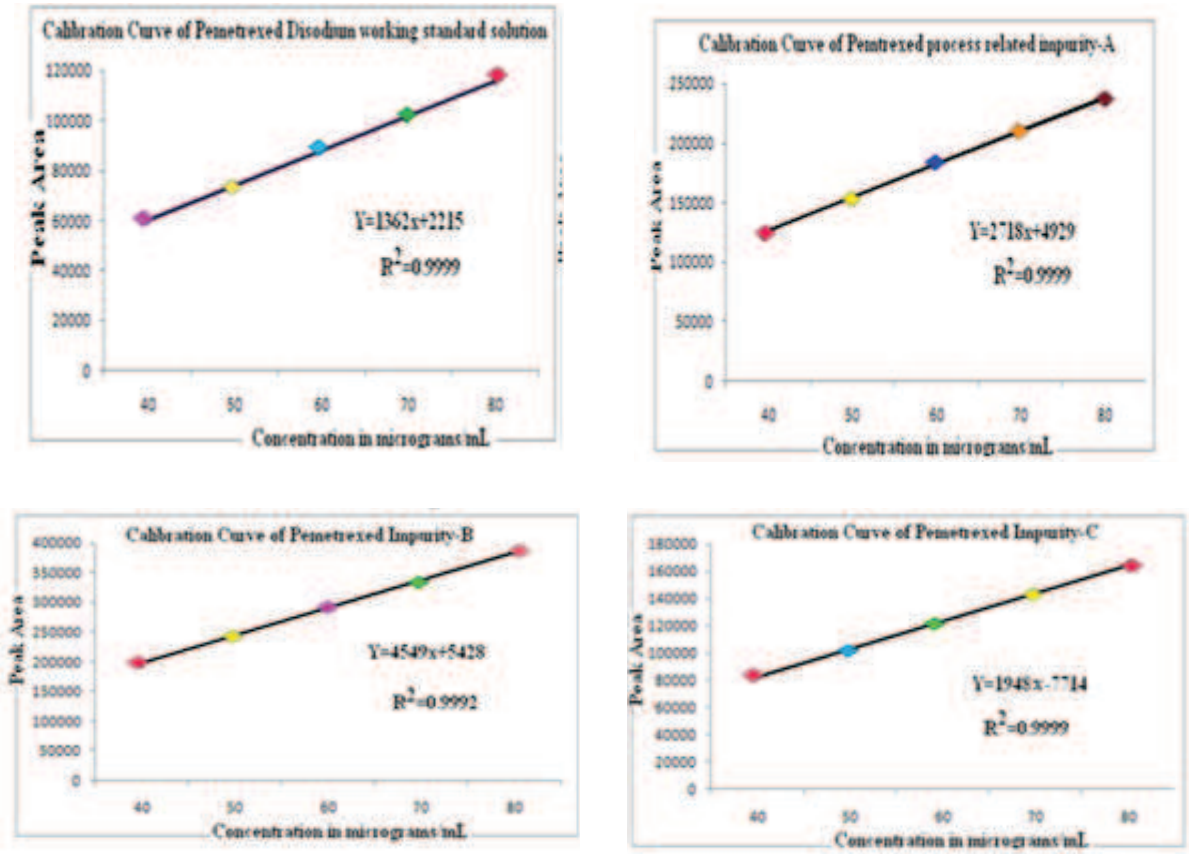
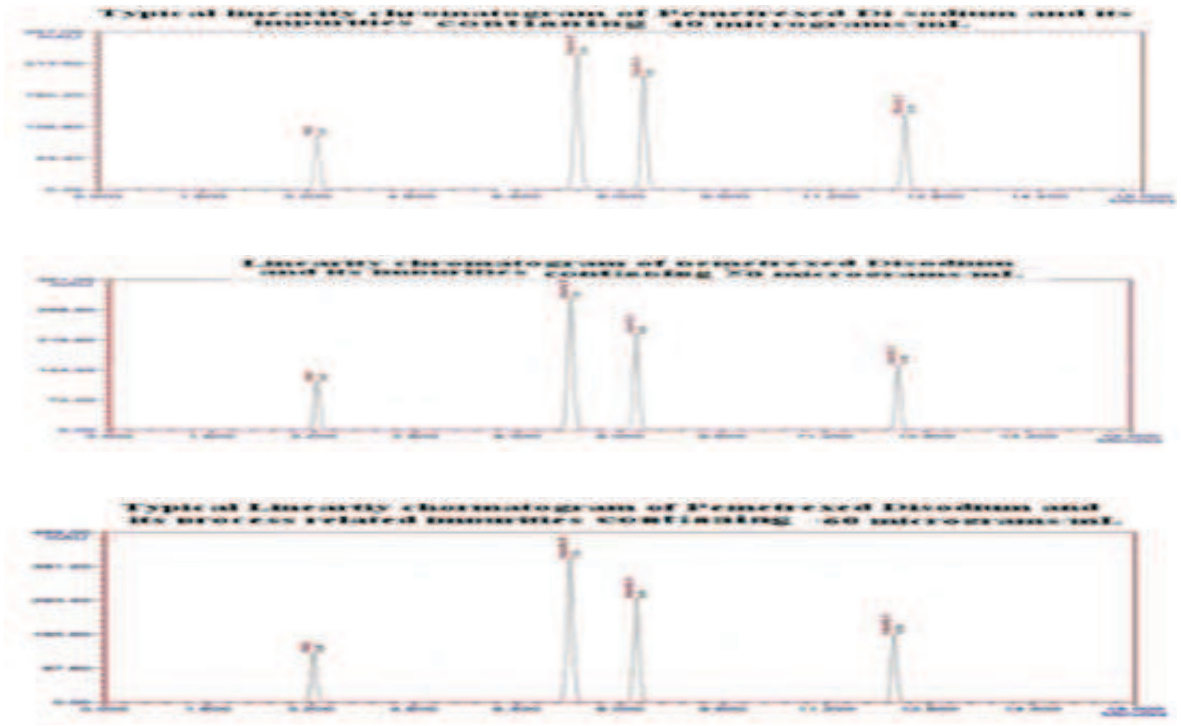


Fig. 4: Calibration Graphs of Pemetrexed Disodium and its Impurities



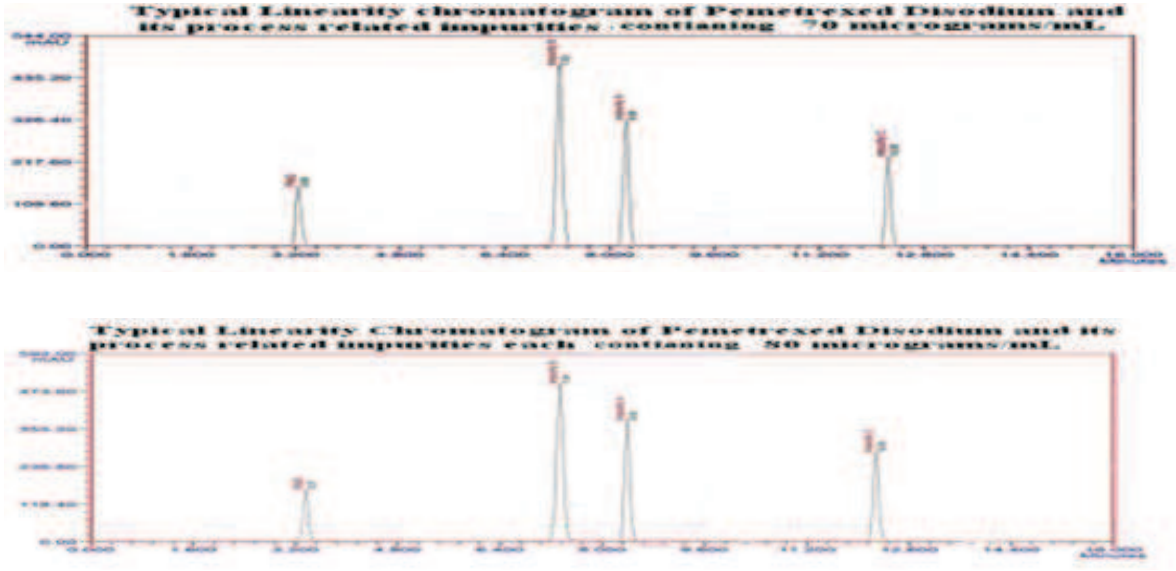


Fig. 5: Linearity Chromatograms of Pemetrexed Disodium and its Impurities

Table 5: Linearity Values of Pemetrexed Disodium and its Impurities

Concentration in µg/mL	Average peak areas			
	Pemetrexed disodium	Impurity-A	Impurity-B	Impurity-C
40	56365	116552	185878	76324
50	70566	145763	232935	98197
60	84805	176820	282687	118358
70	96070	199595	320831	136264
80	111682	230381	369355	154659
Slope	1362	2815	4549	1948
Intercept	2215	4929	5428	7724
Correlation coefficient	0.99999	0.9999	0.9992	0.999

3.6. Accuracy/Recovery: Accuracy of the method by recovery of the impurities were determined by analyzing Pemetrexed disodium sample solutions spiked with each impurity at three different concentration levels ranging from 50%, 75% and 100% in triplicate with respect to specified limit. Recovery values demonstrated that the method was accurate within the desired range.

3.7. Limit of Detection (LOD) and Limit of Quantification (LOQ): The LOD and LOQ for the Pemetrexed disodium and its each impurity were found to be 0.2µg/mL and 0.6µg/mL respectively. LOD chromatogram is shown in Figure 6.

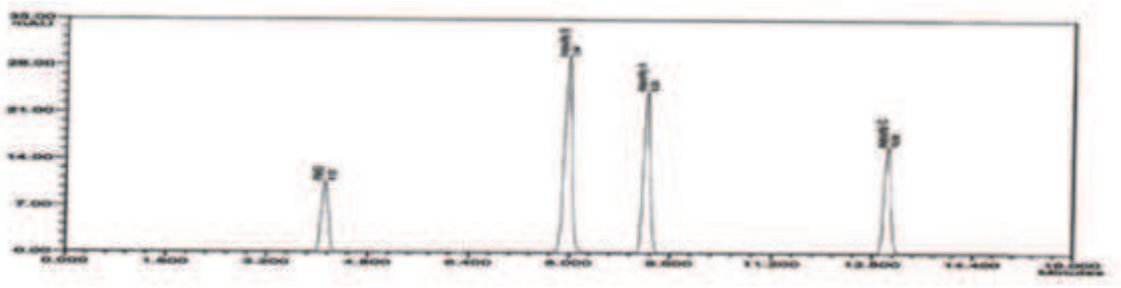


Fig 6: LOD Chromatogram of Pemetrexed Disodium and its Impurities

3.8. Precision at LOQ Level: Standard solutions of Pemetrexed disodium and its impurities were prepared at LOQ concentration of 0.6 µg/mL and injected six times into the system. The % RSD of precision at LOQ level and the results are shown in the Table 6. The Precision chromatogram at LOQ level is shown in Figure 7.

Table 6: Precision Studies at LOQ Level of Pemetrexed and its Impurities

No. of injections of at LOQ level	Pemetrexed disodium	Impurity A	Impurity B	Impurity C
1	19425	44742	74981	32640
2	19126	43935	74303	32672
3	19094	44042	74236	32218
4	19225	44467	75979	32812
5	19181	44486	73610	32473
6	19011	44089	75853	32910
Mean	19177	44293.5	74827	32620.83
Std.Dev.	142.035	316.829	949.57	247.901
% RSD	0.74	0.72	1.27	0.76

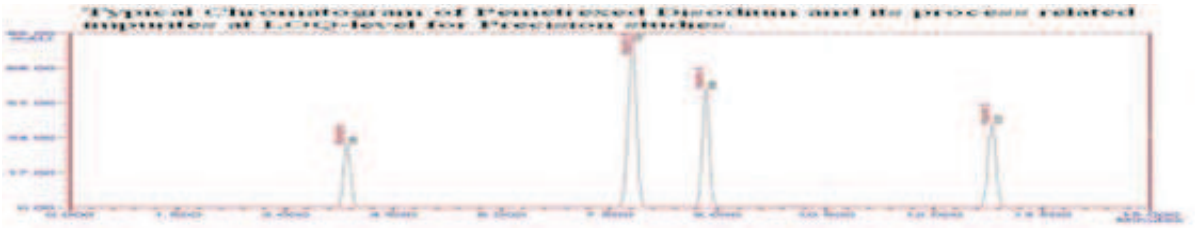


Fig 7: Precision Chromatogram at LOQ level of Pemetrexed Disodium and its Impurities

3.9. Robustness: The parameters selected were different column, mobile phase pH variation and variation in mobile phase composition with organic phase solvent system. The studies indicated that no effect on the determination of related substances and selectivity for the test method is sufficiently robust to carry the quantification of impurities in quality assurance of Pemetrexed disodium. The results of the robustness studies are shown in Table 7.

Table 7: Robustness Study of Pemetrexed Disodium and its Impurities

Parameters studied	Impurity-A		Impurity-B		Impurity-C	
	RT	Peak area	RT	Peak area	RT	Peak area
pH at 5.1	8.25	155135	7.56	331732	12.65	102982
pH at 5.5	8.14	155432	7.51	331801	12.55	102976
Different columns	8.41	155672	7.45	331512	12.41	102435
Diff. HPLC instruments	8.51	155257	7.66	331783	12.75	102972
%RSD		0.23		0.74		0.33

4. Forced Degradation Study: Stability-indicating study of Pemetrexed disodium in bulk form was validated by the proposed method in accordance with ICH guidelines. The drug was subjected to oxidative, acidic, basic, photolytic and thermal stress conditions at zero and 48 hours.

Preparation of Test Solutions for Stress Studies: 1 mL of the primary stock solution contains (500µg/mL) is further diluted with mobile phase up to 25mL in volumetric flask to get the working standard solution of 20µg/mL for forced degradation studies. 0.1N HCl, 0.1N NaOH and 3% H_2O_2 were prepared.

4.1. Acid Stress Studies: To the 20 mL of 20 µg/mL of Pemetrexed disodium degradation sample solution 20 mL of 0.1N hydrochloric acid is added. The zero hour hr sample solution has been prepared by taking immediately 5 mL of the above solution and neutralize with 5 mL of 0.1N sodium hydroxide solution and made up to 25 mL with diluents in volumetric flask. 20 µL of resultant solution was injected into HPLC system. The 48 hr stressed solution was also prepared like zero hr solution and injected into the system. About 30% of drug was degraded. This indicates the significant instability and extremely sensitive nature of Pemetrexed disodium in acid degradation studies.

4.2. Base Stress Studies: To the 20mL of 20 µg/mL of Pemetrexed disodium degradation sample, 20 mL of 0.1N sodium hydroxide is added. The zero hour sample solution has been prepared by taking immediately 5 mL of the above solution and neutralize with 5 mL of 0.1N hydrochloric acid solution and made up to 25 mL with diluents in volumetric flask. 20µL of resultant solution was injected into HPLC system. The 48 hr stressed solution was also prepared like zero hr solution and injected into the system. The typical chromatogram of Pemetrexed disodium in alkaline degradation studies after 48 hours shows that the compound is moderately sensitive in base-stress studies.

4.3. Oxidation Stress Studies: About 20 mL of 20µg/mL of Pemetrexed disodium solution is added to the 20 mL of 3% hydrogen peroxide solution. The zero hour hrs sample solution has been prepared by taking 5 mL of the above solution and diluted up to 25 mL with diluents in

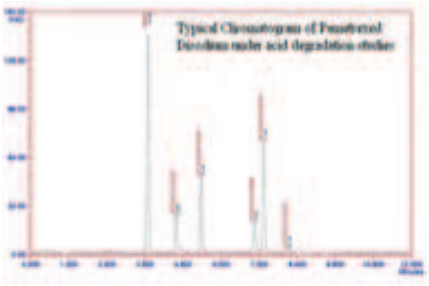
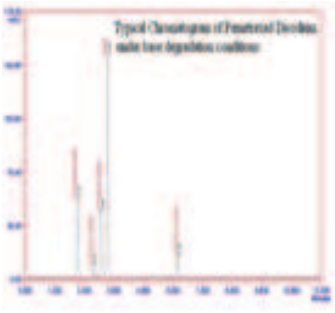
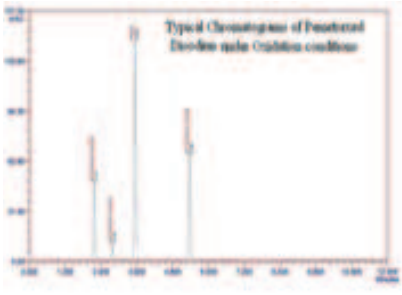
volumetric flask and injected into system. The 48 hr stressed solution was also prepared like zero hr solution and injected into the system. The zero hr sample shows neither degraded Pemetrexed disodium nor shifting in retention time, the 48 hours peroxide induced degradation studies shows that the compound is moderately sensitive to oxidation.

4.4. Thermal Stress Studies: To determine stability of Pemetrexed disodium towards heat 100 mg of sample is kept in petri dish and placed in oven at 80°C up to 48 hours. After 48 hours, the thermally exposed sample at 80°C is used to prepare the test solution at the strength of 20µg/mL with mobile phase and injected in to the liquid chromatography. Approximately 27% of Pemetrexed disodium degraded at 80°C. Thermal degradation studies indicating that the active ingredient is highly sensitive towards heat.

4.5. Photo-Degradation Studies: Photo degradation of the Pemetrexed disodium is determined by the effects of UV- irradiation by keeping the sample in open petri dish at lab light and UV light. After 48 hours, the samples are removed from the UV-light cabinet. The light exposed samples are further used to prepare the testing solution by dilution with mobile phase at the strength of 20µg/mL and injected into the HPLC. Mild degradation was shown towards photo degradation. The forced degradation chromatograms are shown in the Figure 8 and the resulted values are shown in the Table 8.

Table 8: Forced Degradation Values of Pemetrexed Disodium

Stress condition	Color of the solution	RT of Pemetrexed disodium	RT of predominant degradants	% of degradation
Acid 0.1 N HCl	Clear	3.72	4.57, 5.30, 7.04, 7.33 and at 8.03 minutes	30
Base 0.1 N NaOH	Clear	3.348	2.1, 2.7, 3.0, and at 6.1 minutes	35
Oxidation 3% H202	Clear	3.43	2.1, 2.7 and at 6.38 minutes	28
UV Light at 254 nm	Clear	3.42	2.7, 3.0, 4.3, and at 6.1 minutes	39
Heat at 80^0C	Clear	3.379	2.1, 2.7, 3.0, 4.2, 5.2 and at 6.8 minutes	38

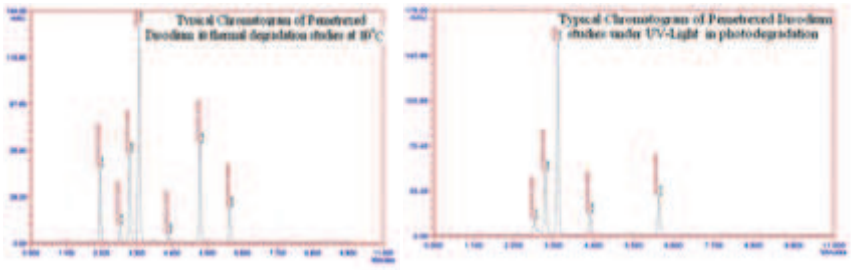


Fig. 8: Typical Chromatograms of Pemetrexed Disodium Under Stress Studies

5. Conclusions: New and simple RP-HPLC method was developed for the simultaneous determination of Pemetrexed disodium and its three related impurities A, B and C in the bulk drug was validated and proved to be reliable, sensitive, accurate, precise and robust. The stress studies are also conducted by subjecting in acid, base hydrolysis, oxidation, heat and UV light. The method was stable and sensitive towards the determination of impurities and it is the first time that such method appears in the literature.

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