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A new validated stability-indicating gradient RP-HPLC method for the determination of pemetrexed disodium and its process related substances

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ABSTRACT

Pemetrexed disodium is used for the treatment of malignant pleural mesothelioma and lung cancer. In the present study a simple stability indicating RP-HPLC method was developed and validated for the determination of Pemetrexed disodium. The process related substances such as Dimer-1 impurity, Dimer-2 impurity, N-Methyl Pemetrexed, Pemetrexed diethyl ester, Alanine derivative of Pemetrexed, DMF derivative of Pemetrexed, Acid intermediate, Oxidation impurity and D-isomer were separated on gradient mode and quantified. Forced degradation studies were performed to prove the specificity. Hypersil BDS C18 100 x 4.6mm, 3µm was used for the separation (at 27°C) with mobile phase mixture consisting of (0.02M sodium dihydrogen phosphate with 0.1% HCOOH and pH 3.8 with dilute sodium hydroxide): Acetonitrile (40:60 v/v) (pH 3.8) with a flow rate of 1.2 mL/min. Methanol: water (1:1) was used as diluent and the eluted compounds were monitored at 240 nm. 0.5-1500 µg/mL with linear regression equation y = 20588x - 9294.1 (R^2=0.9999). The degradation products observed during the forced degradation studies were well resolved from the drug peak and proving that the method is a stability-indicating method. The method was validated as per ICH guidelines.

Keywords: Pemetrexed disodium, RP-HPLC, gradient mode, Related substances, Stability indicating, Validation.

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INTRODUCTION

Pemetrexed disodium was approved for the treatment lung cancer[1-3] either alone or in combination with other drugs. Pemetrexed disodium was quantified by using techniques such as LC-MS/MS[4-5] in human plasma, HPLC in human plasma and urine[6] UPLC[7] in lyophilized parenteral formulation, Chiral liquid chromatography[8-9], HPLC[10-12] methods for related substances[13], spectrophotometric methods[14-15], electrochemical method[16] in the literature. In the present study the authors have developed a simple stability indicating RP-HPLC method for the determination of Pemetrexed disodium and also for the determination of process related substances using Waters Alliance 2695 series HPLC system with 2998 photodiode array detector and the method was validated as per ICH guidelines. The process related substances such as Dimer-1 impurity, Dimer-2 impurity, N-Methyl Pemetrexed, Pemetrexed diethyl ester, Alanine derivative of Pemetrexed, DMF derivative of Pemetrexed, Acid intermediate, Oxidation impurity and D-isomer were separated on gradient mode and quantified. Forced degradation studies were performed to prove the specificity of the method. The chemical structures of Pemetrexed disodium and that of the process related substances were shown in Figure 1.

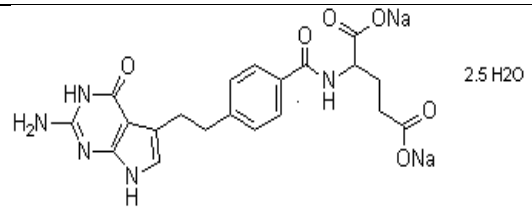
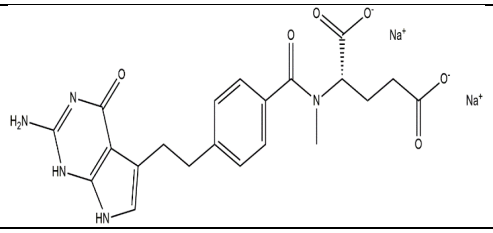
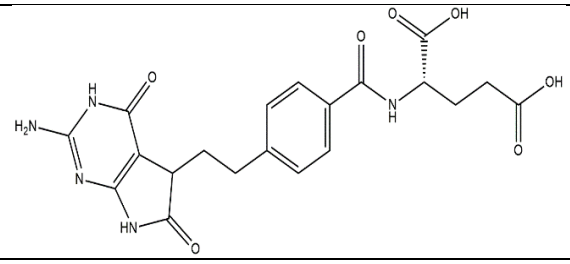
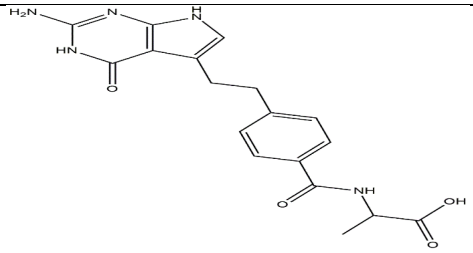
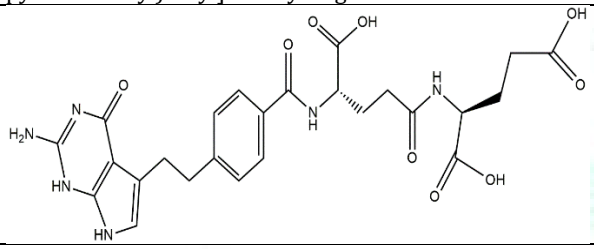
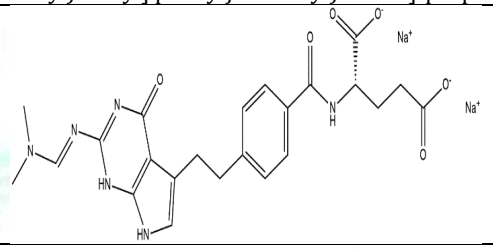
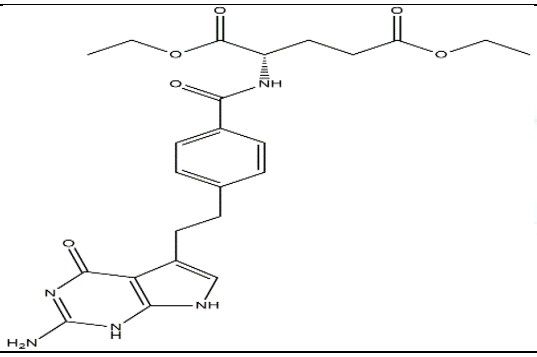
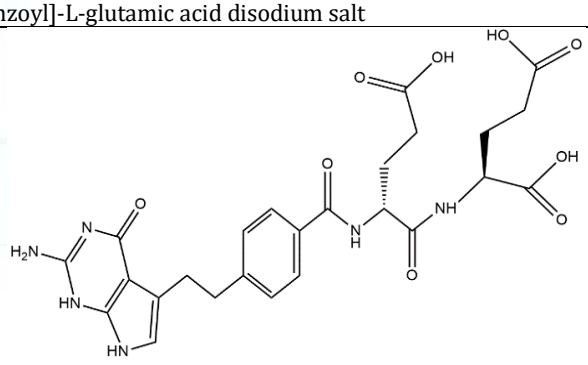
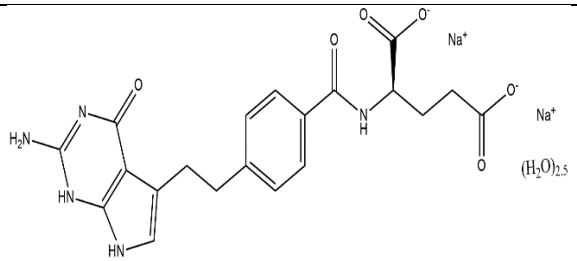
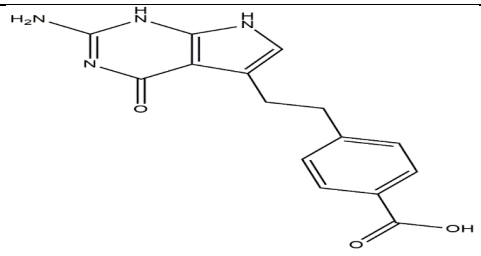
	
(Pemetrexed disodium) Disodium salt of (2*S*)-2-[[4-[2-(2-amino-4-oxo-3,7-dihydropyrrolo [2,3-d] pyrimidin-5-yl) ethyl] benzoyl] amino] pentanedioate	**(N-methyl Pemetrexed)** N-Methyl-[4-[2-(2-Amino-4,7-dihydro-4-oxo-1H-pyrrolo [2,3-d] pyrimidin-5-yl) ethyl] benzoyl]-L-glutamic acid disodium
	
(Oxidation impurity) 4-[2-(2-amino-4,6-dioxo-4,5,6,7-tetrahydro-3*H*-pyrrolo[2,3-*d*] pyrimidin-5-yl)ethyl]benzoyl-L-glutamic acid	**(Alanine derivative of Pemetrexed)** 2-[({4-[2-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo-[2,3-d] pyrimidin-5-yl)- ethyl] phenyl} carbonyl) amino]-propanoic acid
	
(Dimer-1 impurity) N-[4-[2-(2-amino-4,7-dihydro-4-oxo-1H-pyrrolo[2,3-d] pyrimidin-5-yl) ethyl] benzoyl]-L-glutamic acid-γ-dimer	**(DMF derivative of Pemetrexed)** N-[4-[2-(2-{[-(Dimethylamino)-methylidene] amino}-4,7-dihydro-4-oxo-1H-pyrrolo[2,3-d]-pyrimidine-5-yl) ethyl] benzoyl]-L-glutamic acid disodium salt
	
(Pemetrexed diethyl ester) N-[4-[2-(2-Amino-4,7-dihydro-4-oxo-1H pyrrolo[2,3-d] pyrimidin- 5-yl) ethyl] benzoyl]-L-glutamic acid diethyl ester	**(Dimer-2 impurity)** N-[4-[2-(2-amino-4,7-dihydro-4-oxo-1H-pyrrolo[2,3-d] pyrimidin-5-yl) ethyl] benzoyl]-L-glutamic acid-α-dimer
	
(D-Isomer impurity) N-[4-[2-(2-Amino-4, 7-dihydro-4-oxo-1H-pyrrolo [2, 3-d] pyrimidin-5-yl) ethyl] benzoyl]-D-glutamic acid disodium hemi pentahydrate	**(Acid intermediate)** 4- [2-(2-Amino-4,7-dihydro-4-Oxo-1H-pyrrolo [2,3-d] pyrimidin-5-yl) ethyl] benzoic acid

Figure 1: Chemical structures of Pemetrexed disodium and its process related substances

MATERIALS AND METHODS

Pemetrexed disodium and its process related substances were procured from the local pharmaceutical company as gift samples. HPLC grade acetonitrile, methanol, formic acid, TFA, sodium hydroxide, hydrochloric acid, sodium dihydrogen phosphate, ammonium formate and hydrogen peroxide were purchased from Merck (India). Stock solutions containing Pemetrexed disodium and its process related substances were prepared in acetonitrile and diluted using diluent and stored.

Chromatographic conditions

Pemetrexed disodium and its process related substances such as Dimer-1 impurity, Dimer-2 impurity, N-Methyl Pemetrexed, Pemetrexed diethyl ester, Alanine derivative of Pemetrexed, DMF derivative of Pemetrexed, Acid intermediate, Oxidation impurity and D-isomer were separated on gradient mode and quantified using Hypersil BDS C18 (100 x 4.6mm, 3µm) column for separation and quantification using a mixture of mobile phase A (Buffer) consisting of 0.02M sodium dihydrogen phosphate with 0.1% HCOOH (pH adjusted to 3.8 with dilute sodium hydroxide) and mobile phase B consisting of buffer and acetonitrile in the ratio 40:60, v/v with flow rate of 1.2 mL/min (at 27°C). Methanol: water (1:1) was used as diluent. Waters Alliance 2695 series HPLC system with 2998 photodiode array detector and the detector was monitored at 240 nm.

Method validation[17]

Linearity

A series of Pemetrexed disodium solutions (0.5-1500 µg/mL) were prepared spiked with 0.15% process related substances and 10 µL of these solutions were injected in to the HPLC system and the peak area was noted. A series of solutions were also prepared containing Oxidation impurity, Dimer impurity, N-Methyl Pemetrexed, Alanine derivative of Pemetrexed, DMF derivative of Pemetrexed, Acid intermediate and Pemetrexed diethyl ester standard solution at different concentrations at LOQ level, 0.05%, 0.075%, 0.10%,0.12%,0.15%, 0.18%, and 0.225% w.r.t. the working concentration and Pemetrexed disodium standard solution were prepared at different concentrations at LOQ level, 0.05%, 0.075%, 0.10%, 0.12%, 0.15%, 0.18%, 0.225%, 70%, 80%, 90%, 100%, 110%, 120% and 130% w.r.t. the working concentration by performing appropriate dilutions to achieve the targeted concentrations. The linearity graph (calibration curve) was drawn with concentration of solution on the x-axis and mean peak area on the y-axis.

Precision, Accuracy and Robustness

Precision study was performed at its LOQ level. Six replicate sample solutions of Pemetrexed disodium (1.0 mg/mL) containing 0.15% of Oxidation impurity, Dimer impurity, N-Methyl Pemetrexed, Alanine derivative of Pemetrexed, DMF derivative of Pemetrexed, Acid intermediate and Pemetrexed diethyl ester with respect to the sample concentration were prepared and each spiked sample solution was injected, peak area was noted and the % RSD was calculated. Accuracy was studied at LOQ level. Three different sample solutions (1.0 mg/mL) of Pemetrexed disodium containing Oxidation impurity, Dimer impurity, N-Methyl Pemetrexed, Alanine derivative of Pemetrexed, DMF derivative of Pemetrexed and Acid intermediate, Pemetrexed diethyl ester were prepared at LOQ level and injected each solution once in to the system. The peak area of Oxidation impurity, Dimer impurity, N-Methyl Pemetrexed, Alanine derivative of Pemetrexed, DMF derivative of Pemetrexed, Acid intermediate and Pemetrexed diethyl ester % recovery was calculated. Robustness of the method was evaluated by deliberately altering the method conditions from the original method parameters and verifying compliance of the system suitability requirements.

Forced degradation studies[18]

The stability indicating nature of the methods were determined by forced degradation of the drug substance samples using the following conditions such as base hydrolysis, acid hydrolysis, oxidation, thermal degradation and photo degradation. About 500.23 mg of Pemetrexed disodium sample was weighed accurately for the preparation of stock solution and transferred into a 50 mL volumetric flask, dissolved and diluted to volume with diluent and mixed.

Base hydrolysis

For related substances and assay test, 5.0 mL of stock solution was transferred in to a 50 mL volumetric flask and 5.0 mL of 1N NaOH solution was added. This solution was kept for 24 hrs at room temperature and 5 mL of 1N HCl solution was added to this solution and diluted to volume with diluent and mixed. For D-isomer content test, Pemetrexed disodium sample was weighed and transferred into a volumetric flask, 1.0 mL of 0.5N NaOH solution was added, kept for 24 hrs at room temperature and then neutralised with 1.0 mL of 0.5N HCl solution and diluted to volume with diluent and mixed.

Acid hydrolysis

For related substances and assay test, 5.0 mL of stock solution was transferred in to a 50 mL volumetric flask and 5.0 mL of 0.2N HCl solution was added. This solution was kept for 24 hrs at room temperature and 5 mL of 0.2N NaOH solution was added to this solution and diluted to volume with diluent and mixed. For D-isomer content test, Pemetrexed disodium sample was weighed and transferred into a volumetric flask, 1.0 mL of 0.1N HCl solution was added, kept for 24 hrs at room temperature and then neutralised with 1.0 mL of 0.1N NaOH solution and diluted to volume with diluent and mixed.

Oxidation

For related substances and assay test, 5.0 mL of stock solution was transferred in to a 50 mL volumetric flask and 5.0 mL of 1.0% H_2O_2 solution was added. This solution was kept for 48 hrs at room temperature and after 48 hrs diluted to volume with diluent and mixed. For D-isomer content test, Pemetrexed disodium sample was weighed and transferred into a volumetric flask, 1.0 mL of 0.5% H_2O_2 solution was added, kept for 48 hrs at room temperature and after 48 hrs diluted to volume with diluent and mixed.

Photo degradation

About 0.5g of Pemetrexed disodium sample was weighed and transferred in to a petri dish and kept in photo stability chamber (1.2 million lux hours and 200 Watt Hrs/Sq.Mtr). For related substances and assay test, about 50 mg of photo degraded sample was transferred into a 50 mL of volumetric flask, dissolved and diluted to volume with diluent and mixed. For D-isomer content test, about 50 mg of photo degraded sample was transferred into a 10 mL of volumetric flask, dissolved and diluted to volume with diluent and mixed.

Thermal degradation

About 0.5g of Pemetrexed disodium sample was weighed and transferred in to a petri dish and kept in oven at 60°C for 9 days. For related substances and assay test, about 50 mg of thermal degraded sample was transferred into a 50 mL of volumetric flask, dissolved and diluted to volume with diluent and mixed. For D-isomer content test, about 50 mg of thermal degraded sample was transferred into a 10 mL of volumetric flask, dissolved and diluted to volume with diluent and mixed.

Assay of Pemetrexed disodium injection

Pemetrexed disodium is available with brand names ALIMTA (Label claim: 100 mg/vial & 500 mg); (Eli Lilly and Company, India) PEXATE (Label claim: 100 mg/vial) (Miracalus Pharma Pvt Ltd), GIOPEM (Label claim: 100 mg/vial & 500 mg/vial) (GLS Pharma Ltd) as solution for injection. Two different brands were chosen and extracted with the mobile phase for the API and diluted as per the requirement and the percentage purity of Pemetrexed disodium was determined.

RESULTS

A simple and specific stability indicating gradient RP-HPLC method was developed and validated for the separation and quantification of Pemetrexed disodium and its related substances using Hypersil BDS C18 (100 x 4.6 mm, 3µm) column with flow rate 1.2 ml/min within a run time of 55 mins.

Method optimization

During optimization different columns and mobile compositions were used in trials with different flow rates and finally the method was optimized. The mobile phase A consists of a buffer solution containing 0.02M sodium dihydrogen phosphate with 0.1% HCOOH maintaining pH 3.8 (adjusted with dilute sodium hydroxide). The mobile phase B consists of a mixture of buffer and acetonitrile in the ratio 40:60, v/v and the detector was monitored at 240 nm. The observations and conclusions recorded during the trial runs were shown in Table 1. The chromatograms obtained during the trials as well as the optimized conditions were shown in Figure 2.

Table 1: Method optimization

Trial	Mobile phase (v/v) / Flow rate (ml/min) / Detection wavelength (nm)	Diluent	Gradient program (T/%B)	Observations and conclusions	Figure
1 Inertsil ODS-2V (250 x 4.6mm, 5µm) column	(Buffer:Acetonitrile 90: 10):: Buffer:Acetonitrile 10: 90)/ 1.5 / 240	Water: Acetonitrile: 2% aq. TFA (90:10:0.1)	0/8, 15/15, 20/25, 25/25, 26/8, 40/8	Base line drift was observed and some of the impurities were closely eluted with the main peak and no better resolution between the peaks.	2A 2B
2 Selection of diluent	(Buffer:Acetonitrile, 90: 10)::Buffer:Acetonitrile 10: 90) / 1.5 / 240	Acetonitrile: water (1:1) Methanol: water (1:1)	”	Pemetrexed peak shape was distorted in presence of Acetonitrile i.e. peak splitting was observed Therefore diluent was changed.	2C 2D
2 Selection of wavelength	(Buffer:Acetonitrile 90: 10):: Buffer:Acetonitrile 10: 90)/ 1.5 / 240	Methanol: water (1:1)	”	Base line drift was more at 225 nm than at 240 nm. Unknown impurities were observed at 240 nm.	2E 2F
4 Selection of buffer and pH	0.02 M Ammonium formate buffer was used with pH 2.5, 2.8 and 4.5	”		Optimum pH 2.8 was selected as all the peaks were resolved.	2G 2H 2I
5 Mobile phase composition optimization	(0.02M Ammonium formate (pH 2.8 adjusted with formic acid): (Buffer: Acetonitrile [100: 920:70)] / 1.2 / 240	”	0/20, 5/20, 20/50, 5/50, 50/80, 5/80, 56/20, 60/20	Two impurities were eluted closely i.e. at 4.2 min and 4.5 min. Impurity observed at 4.2 min is a process related impurity.	2J

6 Flow rate, Mobile phase B and gradient program were changed	Buffer: (Buffer, Methanol and Acetonitrile) [100: (25:20:55)] / 1.5 / 240	”	0/20, 5/20, 15/25, 0/25, 20/60, 0/60, 45/80, 6/20, 50/20	Impurity observed at 9.2 min is a combination of two peaks. and two peaks at 9.8 and 10.5 min Impurities at 4.2 and 4.5 min were not separated completely.	2K
7 Buffer strength was enhanced from 0.02 M to 0.05 M	”	”	”	Impurity observed at 4.2 and 4.5 min were well resolved but Pemetrexed peak shape was not symmetric. Resolution was 1.2 and theoretical plates were 17000.	2L
8 Trifluoro acetic acid (TFA) was introduced in to mobile phase.	0.05M Ammonium formate with 0.1% TFA (pH adjusted to 2.8 with formic acid)	”	”	The Dimer-2 impurity was co-eluted along with Pemetrexed peak. Resolution was improved (i.e. from 1.2 to 2.1) and theoretical plates were 18650.	2M
9 Pemetrexed was spiked with 0.15% of all impurities.	0.05M Ammonium formate with 0.1% TFA (pH adjusted to 2.8 with HCOOH)	”	0/17, 20/17, 50/80, 25/17, 60/17	All impurities were well resolved. Resolution between Pemetrexed and Dimer-2 obtained was 2.15.	2N
10 Pemetrexed was spiked with 0.15% of all impurities.	Selection of Hypersil BDS C18 (100 x 4.6 mm, 2µm) column / 1.2	”	0/15, 15/15, 45/50, 50/15, 55/15	Resolution between Pemetrexed and Dimer-2 was 2.20 but baseline drift was observed.	2O
11 Volatile buffer was replaced with phosphate buffer	Buffer [0.02M Sodium dihydrogen phosphate with 0.1% HCOOH (pH adjusted to 3.8 with dil NaOH)]: [Buffer: Acetonitrile, 40:60]	”	”	Baseline drift was reduced and resolution was 2.06. **(Method optimized)**	2P

Method validation

Linearity

Pemetrexed disodium has shown linearity over the concentration range 0.5-1500 µg/mL with linear regression equation y = 20588x - 9294.1 (R^2=0.9999) (Table 2) and the calibration curve was shown in Figure 3. Good linearity response was also obtained for Pemetrexed disodium peak over the concentration ranges of LOQ to 0.225% and LOQ to 130% w.r.t. the working concentration (Table 3). The linearity of related substances was shown in Table 4 and the corresponding regression equations along with the relative response factors were shown in Table 5. The method covered the range 0.1668 - 2.2751 µg/mL for Oxidation impurity, 0.2704 - 2.2640 µg/mL for Dimer impurity, 0.1811 - 2.1849 µg/mL for N-Methyl Pemetrexed, 0.1674 - 2.2757 µg/mL for Alanine derivative of Pemetrexed, 0.0997 - 2.2604 µg/mL for DMF derivative of Pemetrexed, 0.0989 - 2.2443 µg/mL for Acid intermediate and 0.1447 - 2.2716 µg/mL for Pemetrexed diethyl ester. Good linearity response was obtained for Pemetrexed disodium and its related substances and the correlation coefficient of linear regression equations was not less than 0.98 for each Impurity and Pemetrexed disodium.

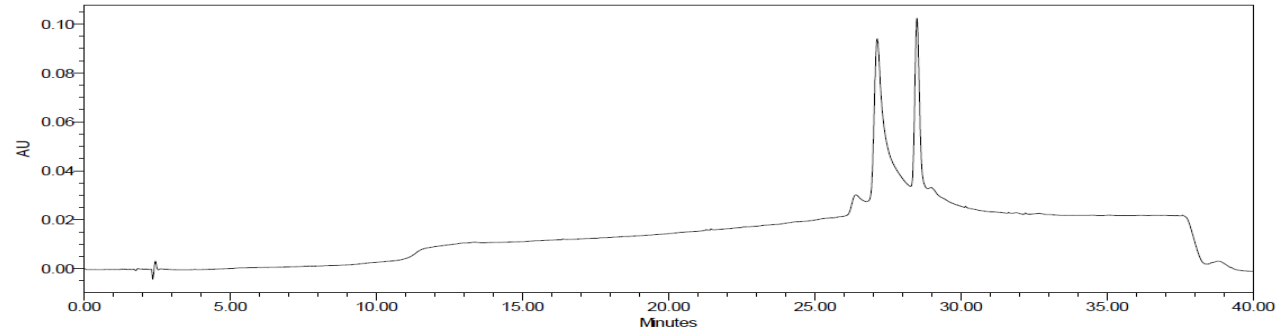


Figure 2A: Chromatogram of blank

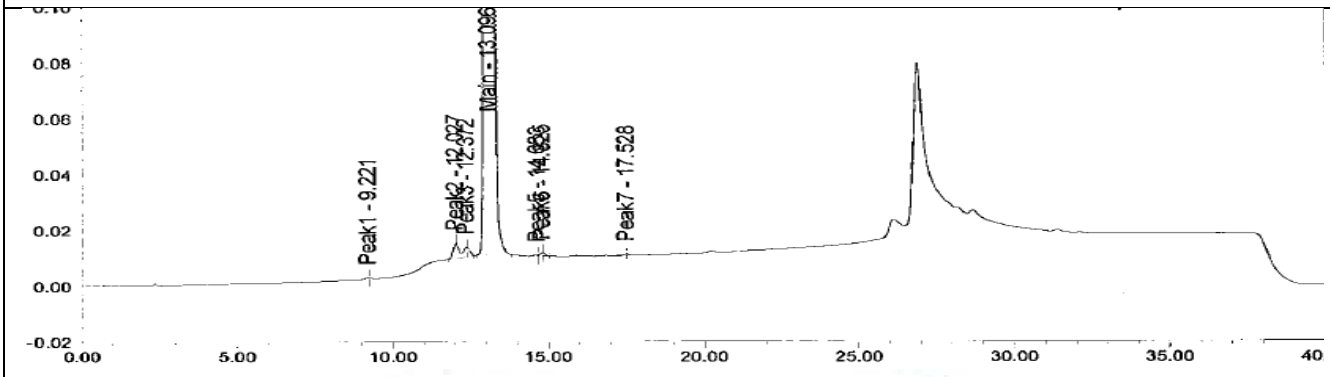


Figure 2B: Chromatogram of Premetrexed (Trial 1)

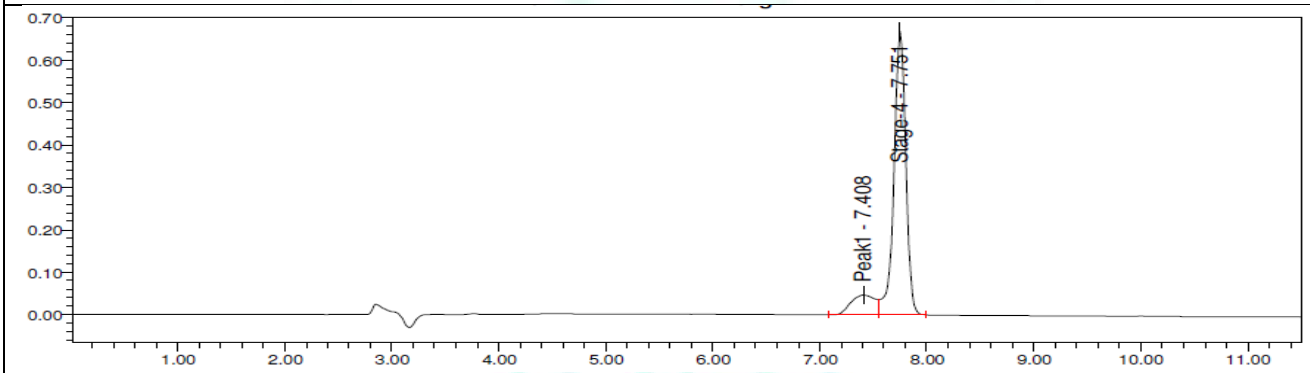


Figure 2C: Chromatogram of Premetrexed in Acetonitrile and water (1:1) as diluent (Trial 2)

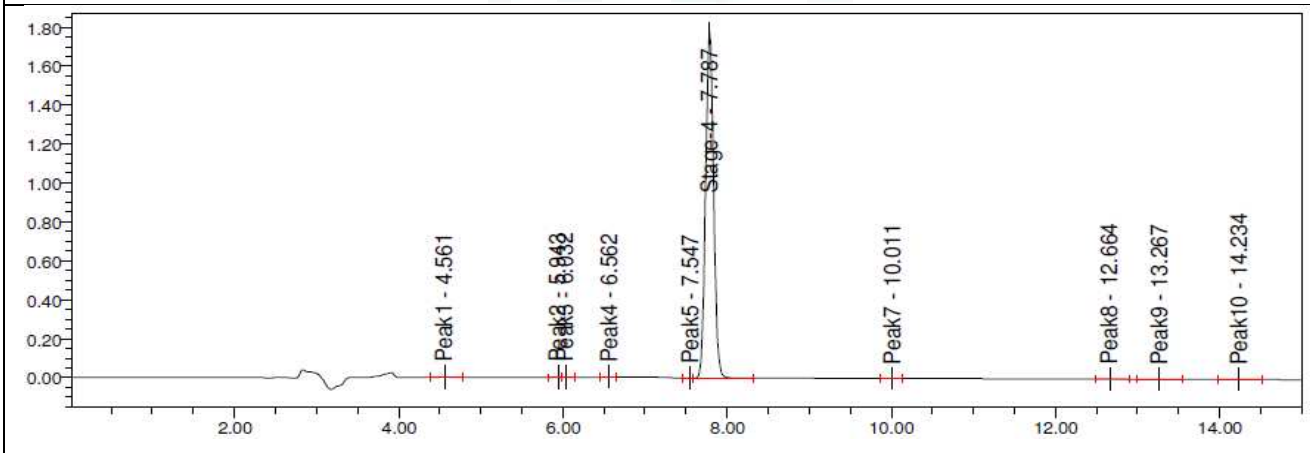


Figure 2D: Chromatogram of Premetrexed in Methanol and water (1:1) as diluent (Trial 2)

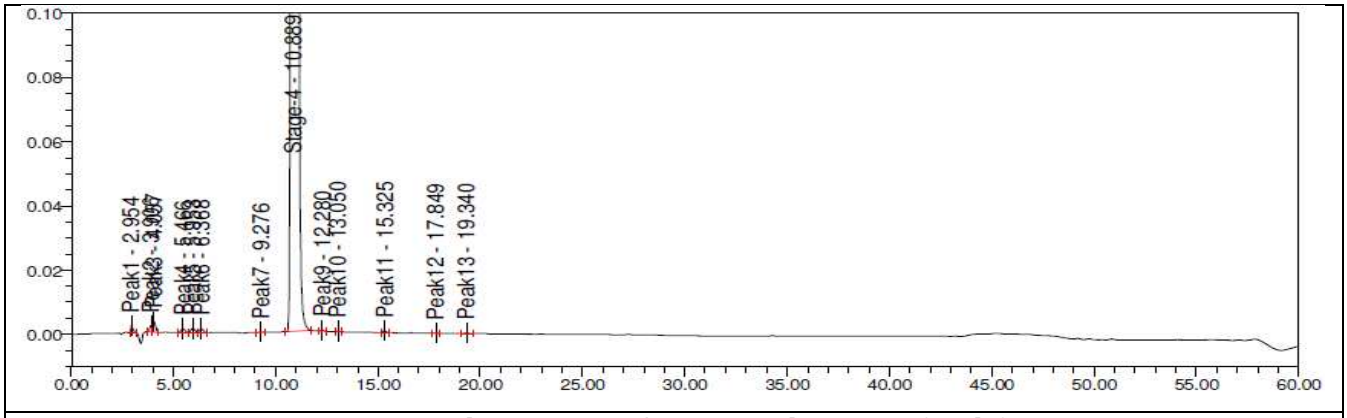


Figure 2E: Chromatogram of Premetrexed at 240 nm (Trial 2)

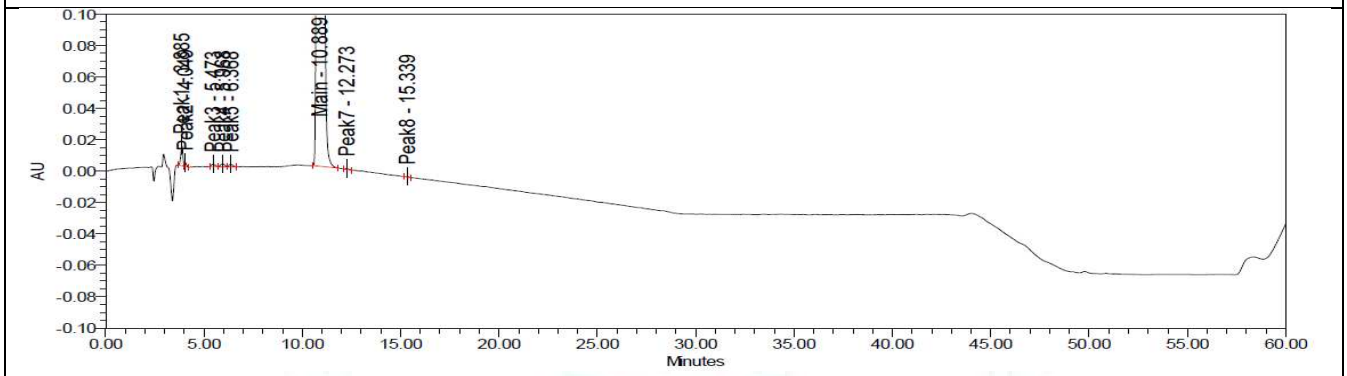


Figure 2F: Chromatogram of Premetrexed at 225 nm (Trial 2)

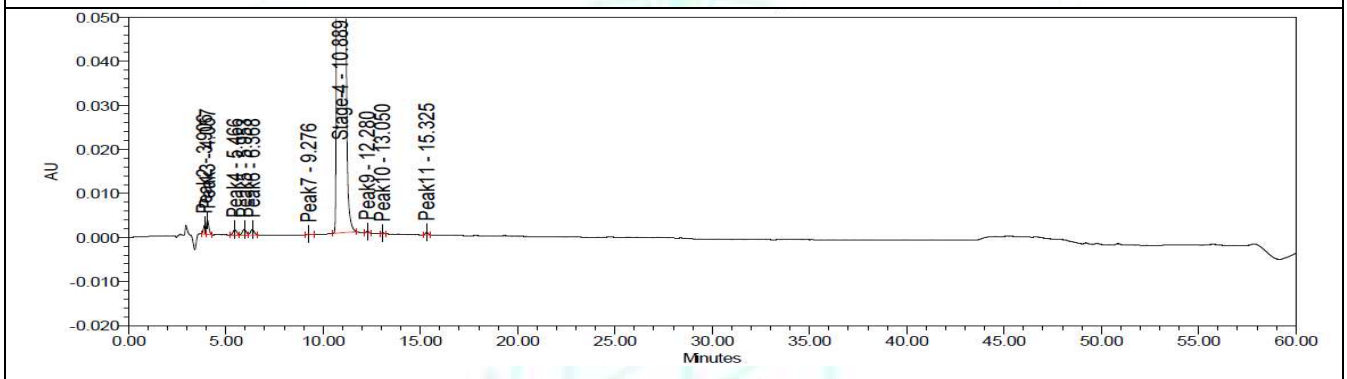


Figure 2G: Chromatogram of Premetrexed at buffer pH 4.5 (Trial 4)

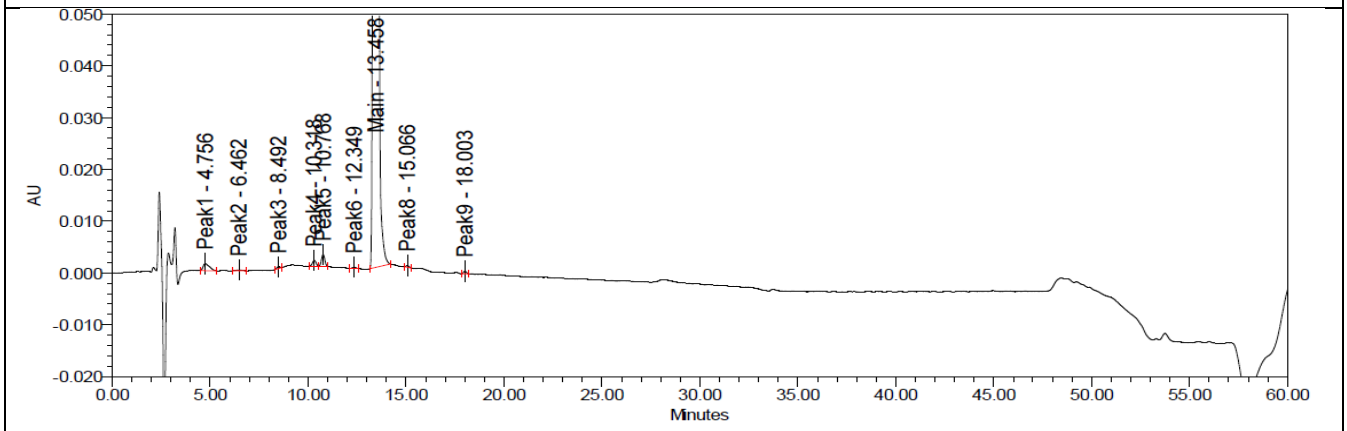


Figure 2H: Chromatogram of Premetrexed at buffer pH 2.5 (Trial 4)

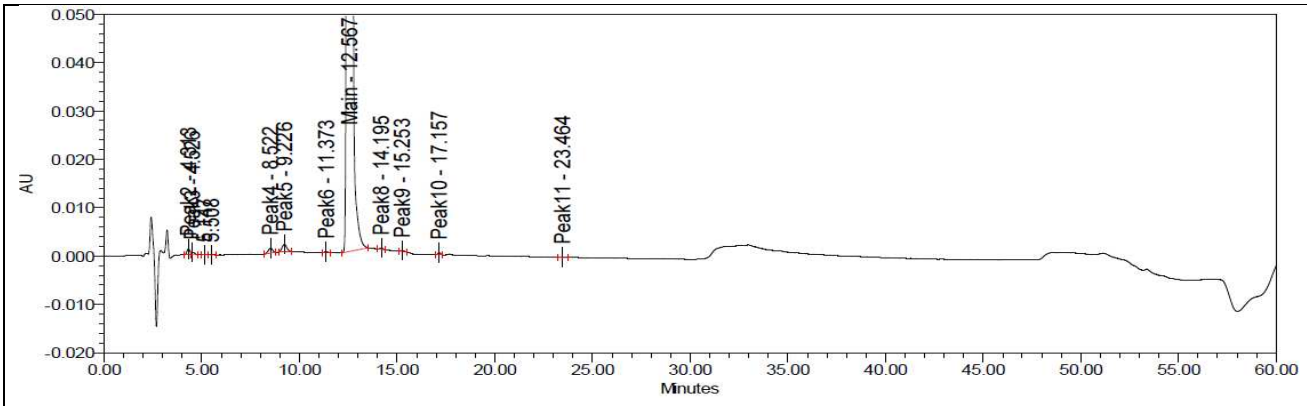


Figure 2I: Chromatogram of Premetrexed at buffer pH 2.8 (Trial 4)

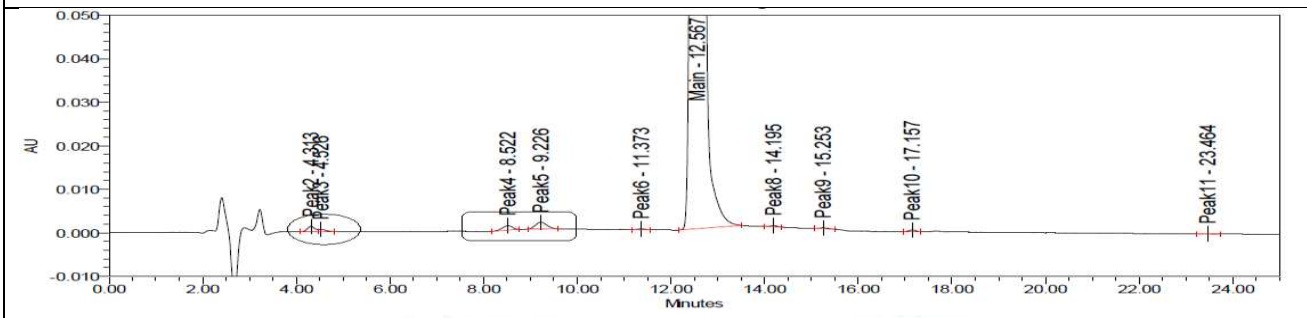


Figure 2J: Chromatogram of Premetrexed (Mobile phase composition changed) (Trial 5)

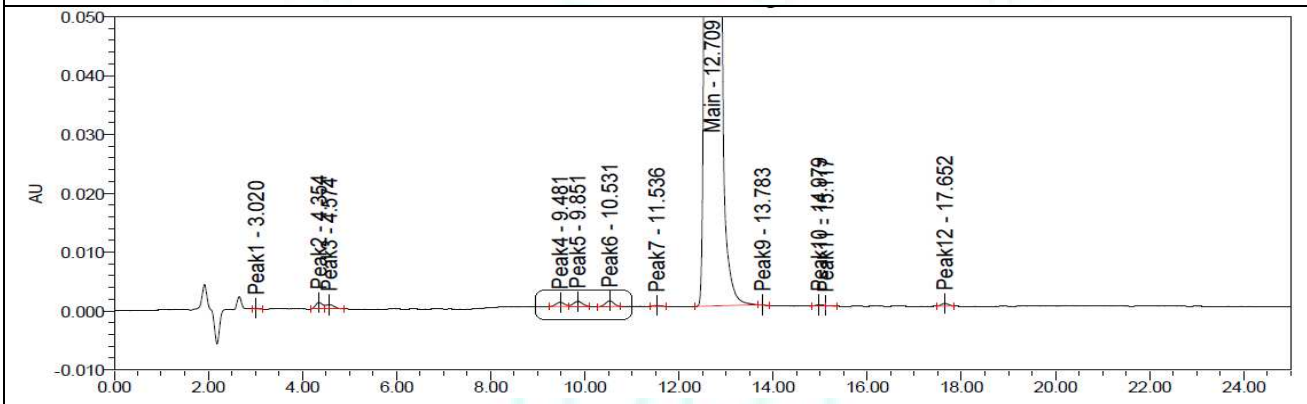


Figure 2K: Chromatogram of Premetrexed (Flow rate, mobile phase composition and gradient program changed) (Trial 6)

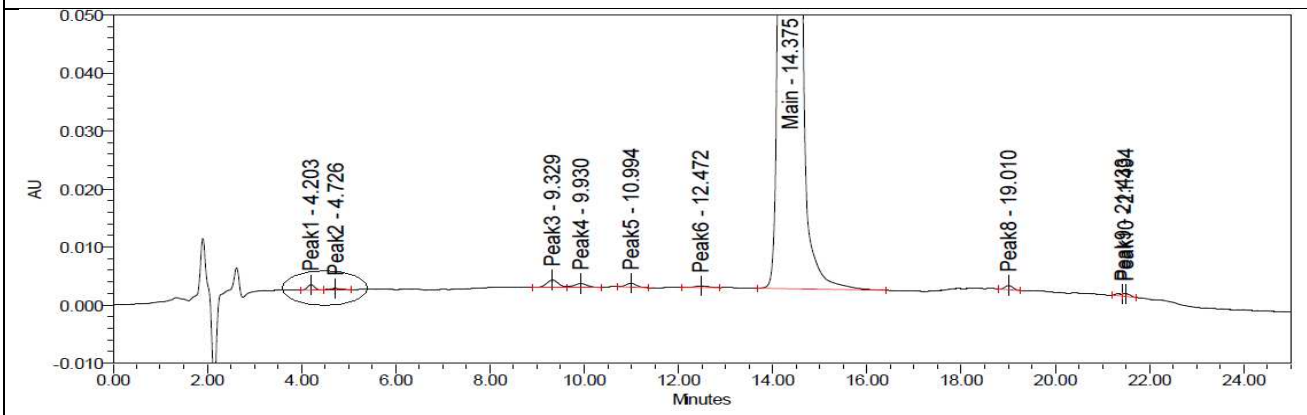


Figure 2L: Chromatogram of Premetrexed (Buffer strength enhanced from 0.02 to 0.05 M (Trial 7)

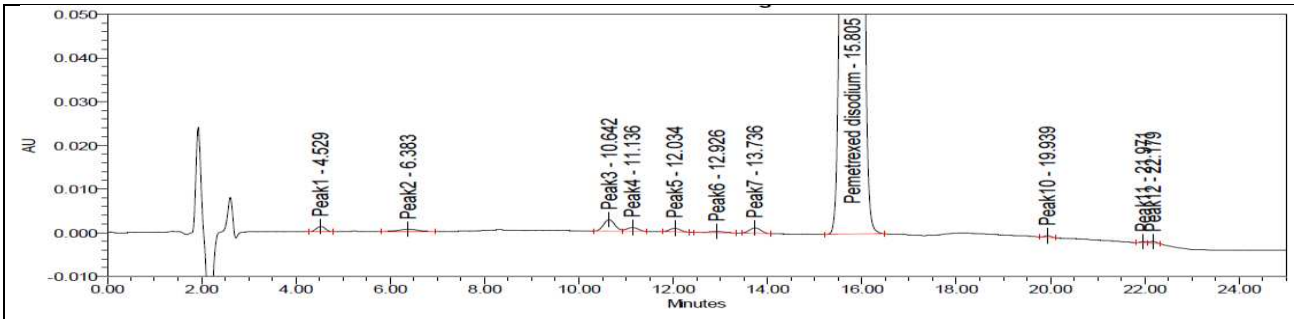


Figure 2M: Chromatogram of Premetrexed (TFA introduced in to mobile phase) (Trial 8)

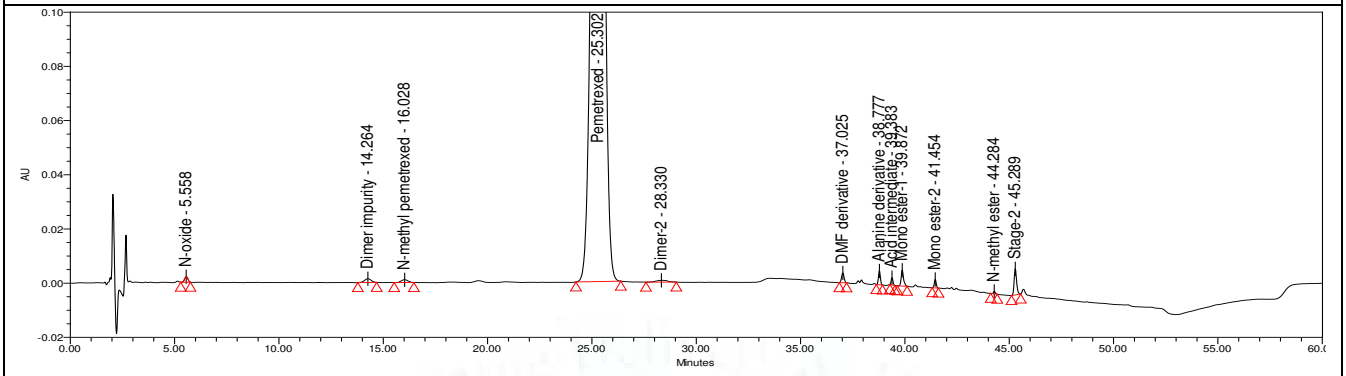


Figure 2N: Chromatogram of Premetrexed spiked with 0.15% of all impurities (Gradient program modified) (Trial 9)

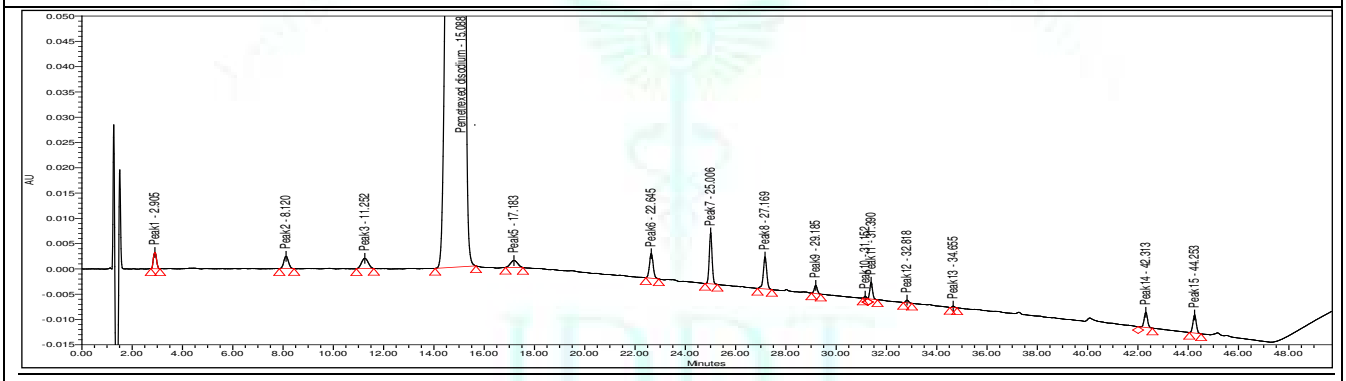


Figure 2O: Chromatogram of Premetrexed spiked with 0.15% of all impurities (Column and flow rate changed) (Trial 10)

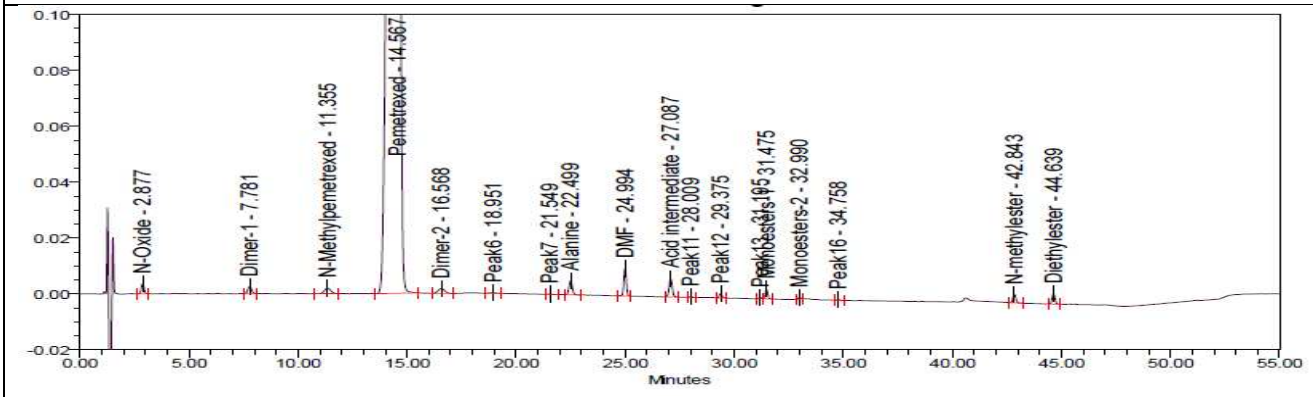


Figure 2P: Chromatogram of Premetrexed spiked with 0.15% of all impurities (Volatile buffer replaced with phosphate buffer) (Trial 11) (Method optimized)

Table 2: Linearity of Pemetrexed disodium

Conc. (µg/mL)	Mean peak area
0.5	10802
1	22174
1.5	22129
5	108968
50	1021849
100	2049502
200	4099121
400	8201269
500	10224220
800	16400710
1000	20484088
1200	24952028
1500	20787406
Slope	20588
y-intercept	-9294.1
Correlation coefficient	0.9999

Table 3: Linearity results of Pemetrexed disodium

LOQ to 0.225% w.r.t working conc.	
Conc. (µg/mL)	**Peak area**
0.2222	6594
0.4356	11600
0.6600	17375
0.8843	23222
1.0559	27279
1.3199	34191
1.5839	41159
1.9799	51241
Regression equation	25511+ 620 (r^2 = 0.9999)
LOQ to 130% w.r.t working conc.	
626.2505	14379381
715.7149	16562547
805.1792	18670056
894.6436	20593974
984.1080	22666881
1073.5723	24529007
1163.0367	26683259
Regression equation	22982 + 12315 (r^2 = 1.0000)

Table 4: Linearity of related substances of Pemetrexed disodium

Oxidation impurity		Dimer impurity		N-methyl Pemetrexed		Alanine derivative of Pemetrexed		DMF derivative of Pemetrexed		Acid intermediate		Pemetrexed diethyl ester	
Conc. (µg/mL)	**Peak area**	**Conc. (µg/mL)**	**Peak area**	**Conc. (µg/mL)**	**Peak area**	**Conc. (µg/mL)**	**Peak area**	**Conc. (µg/mL)**	**Peak area**	**Conc. (µg/mL)**	**Peak area**	**Conc. (µg/mL)**	**Peak area**
0.1668	2692	0.2704	4050	0.1811	4418	0.1674	4106	0.0997	2665	0.0989	3442	0.1447	3216
0.5005	8680	0.4981	7290	0.4807	11764	0.5006	12182	0.4973	13407	0.4937	16975	0.4998	10711
0.7584	13131	0.7547	11268	0.7283	18084	0.7586	19222	0.7535	20377	0.7481	25615	0.7572	16299
1.0162	17629	1.0112	14894	0.9759	24315	1.0165	26011	1.0096	27558	1.0025	33731	1.0146	21782
1.2134	20898	1.2074	17349	1.1653	28790	1.2137	30709	1.2055	32956	1.1970	40213	1.2115	25853
1.5167	26244	1.5093	22579	1.4566	36362	1.5171	39088	1.5069	41608	1.4962	51089	1.5144	32472
1.8200	31339	1.8112	26747	1.7479	43899	1.8205	46473	1.8083	49972	1.7954	61420	1.8173	39028
2.2751	38907	2.2640	34089	2.1849	55105	2.2757	57815	2.2604	61055	2.2443	77226	2.2716	48640

Table 5: Linearity (Regression equations) of related substances

Analyte name	Regression equation	Relative response factor (RRF)
Oxidation impurity	Y = 17171x + 49 (0.9999)	1.63
Dimer impurity	Y = 15002x - 206 (0.9996)	1.90
N-Methyl Pemetrexed	Y = 25299x - 369 (0.9999)	1.11
Alanine derivative of Pemetrexed	Y = 25648x - 269 (0.9999)	1.05
DMF derivative of Pemetrexed	Y = 27326x - 24 (0.9998)	0.93
Acid intermediate	Y = 34316x - 218 (0.9999)	0.74
Pemetrexed diethyl ester	Y = 21389x + 69 (1.0000)	1.19

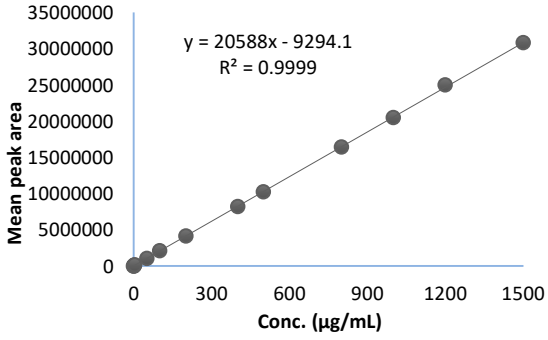


Figure 3: Calibration curve of Pemetrexed disodium

Limit of Detection and Limit of Quantitation (LOD and LOQ)

LOD and LOQ were established by injecting diluted solutions having known concentration of Pemetrexed disodium, Oxidation impurity, Dimer impurity, N-Methyl Pemetrexed, Alanine derivative of Pemetrexed, DMF derivative of Pemetrexed, Acid intermediate and Pemetrexed diethyl ester to obtain a signal to noise ratio of greater than or equal to 3 and 10 for LOD and LOQ respectively (Table 6).

Table 6: LOD and LOQ of Pemetrexed disodium and its related substances

Analyte name	LOD	LOQ
Pemetrexed disodium	0.0738	0.2239
Oxidation impurity	0.0550	0.1668
Dimer impurity	0.0892	0.2704
N-Methyl Pemetrexed	0.0597	0.1811
Alanine derivative of Pemetrexed	0.0552	0.1674
DMF derivative of Pemetrexed	0.0322	0.0977
Acid intermediate	0.0326	0.0989
Pemetrexed diethyl ester	0.0477	0.1447

Precision at LOQ level

The RSD at LOQ level was obtained as 3.1% for Pemetrexed disodium, 8.5% for Oxidation impurity, 2.9% for Dimer impurity, 3.1% for N-Methyl Pemetrexed, 2.5% for Alanine derivative of Pemetrexed, 3.2% for DMF derivative of Pemetrexed, 4.9% for Acid intermediate and 2.7% for Pemetrexed diethyl ester indicating that the acceptance criteria was achieved. Acceptable criteria mean that the RSD at LOQ level should not be more than 15.0% for each analyte (Table 7).

Table 7: Precision study of Pemetrexed disodium and its related substances

S. No.	Peak area							
	Pemetrexed disodium	Oxidation impurity	Dimer impurity	N-Methyl Pemetrexed	Alanine derivative of Pemetrexed	DMF derivative of Pemetrexed	Acid intermediate	Pemetrexed diethyl ester
1	6844	2873	3858	4216	4292	3132	3352	3110
2	6656	2426	3871	4275	4134	3080	3478	3201
3	6542	2849	4011	4528	4044	3133	3131	3022
4	6858	2434	4068	4541	4266	3201	3049	3122
5	6760	2948	4150	4310	4183	3099	3284	3196
6	6318	2633	4077	4351	4049	3358	3367	3267
Mean	6663	2694	4006	4370	4161	3167	3277	3153
RSD	3.1%	8.5%	2.9%	3.1%	2.5%	3.2%	4.9%	2.7%

Accuracy

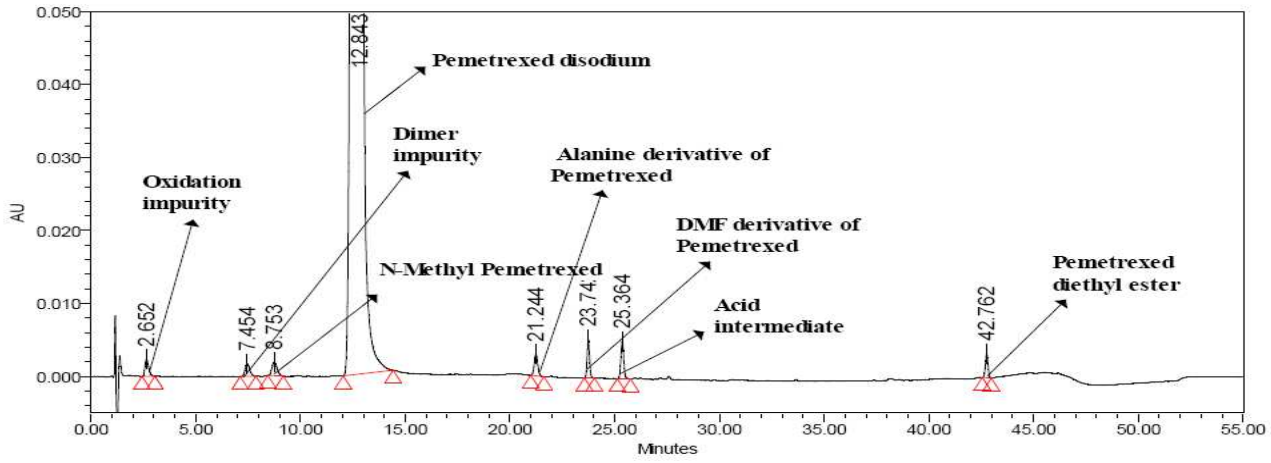
The recovery obtained (at LOQ level) was in the range of 100.8% - 113.7% for Oxidation impurity, 109.5% - 112.6% for Dimer impurity, 105.0% - 112.2% for N-Methyl Pemetrexed, 111.5% - 114.1% for Alanine derivative of Pemetrexed, 99.3% - 99.8% for DMF derivative of Pemetrexed, 114.4% - 116.1% for Acid intermediate and 114.2% - 115.4% for Pemetrexed diethyl ester indicating that the acceptance criteria was fulfilled (Recovery should be within the range of 70.0 – 130.0%) (Table 8).

Table 8: Accuracy of process related substances

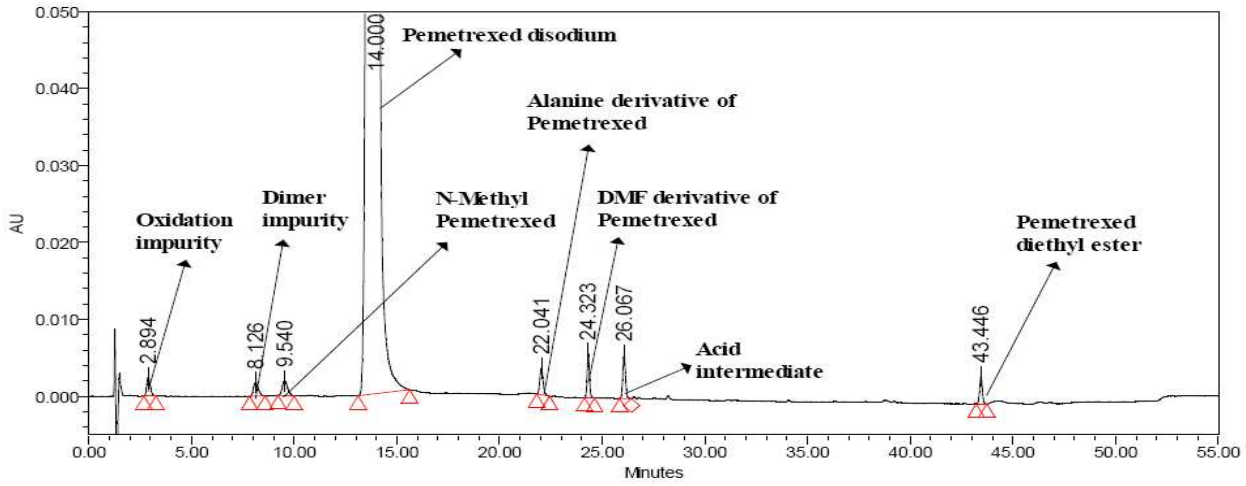
Name	Workup	Amount added (µg/mL)	Amount obtained (µg/mL)	% Recovery	% Mean recovery
Oxidation impurity	1	0.1834	0.2085	113.7	109.0
	2		0.2061	112.4	
	3		0.1848	100.8	
Dimer impurity	1	0.3025	0.3406	112.6	110.6
	2		0.3313	109.5	
	3		0.3315	109.6	
N-Methyl Pemetrexed	1	0.1911	0.2233	112.2	109.7
	2		0.2091	105.0	
	3		0.2230	112.0	
Alanine derivative	1	0.1774	0.1978	111.5	112.7
	2		0.1994	112.4	
	3		0.2024	114.1	
DMF derivative	1	0.0997	0.0995	99.8	99.6
	2		0.0990	99.3	
	3		0.0994	99.7	
Acid intermediate	1	0.0989	0.1131	114.4	115.4
	2		0.1143	115.6	
	3		0.1148	116.1	
Pemetrexed diethyl ester	1	0.1447	0.1653	114.2	115.0
	2		0.1668	115.3	
	3		0.1670	115.4	

Robustness

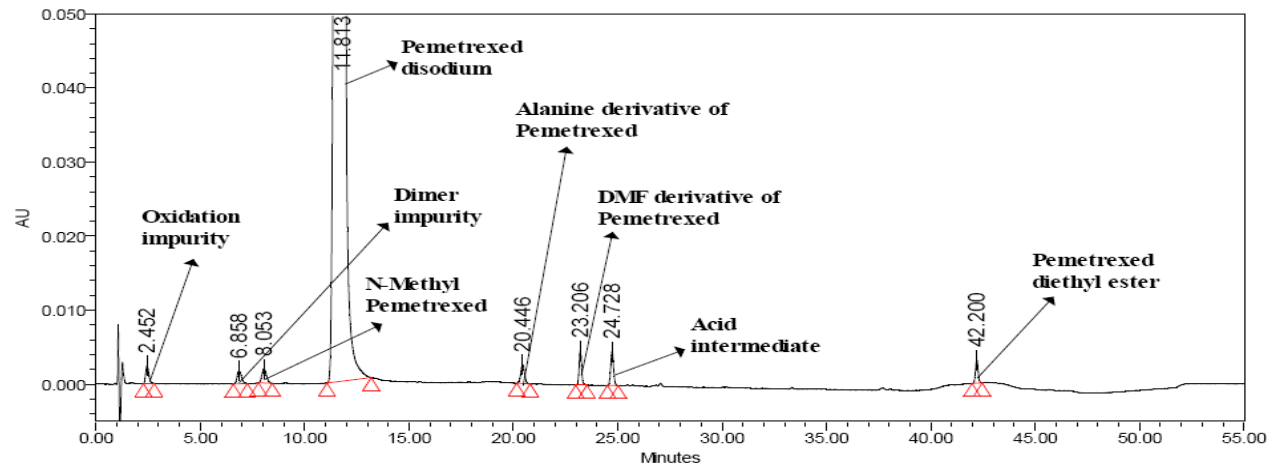
The effect of flow rate, column temperature, buffer pH, mobile phase composition on system suitability were summarized in Table 9. Pemetrexed disodium was well separated from the related substances such as acid intermediate, alanine derivative of Pemetrexed, DMF derivative of Pemetrexed and Pemetrexed diethyl ester to prove that the method is specific (Figure 4).



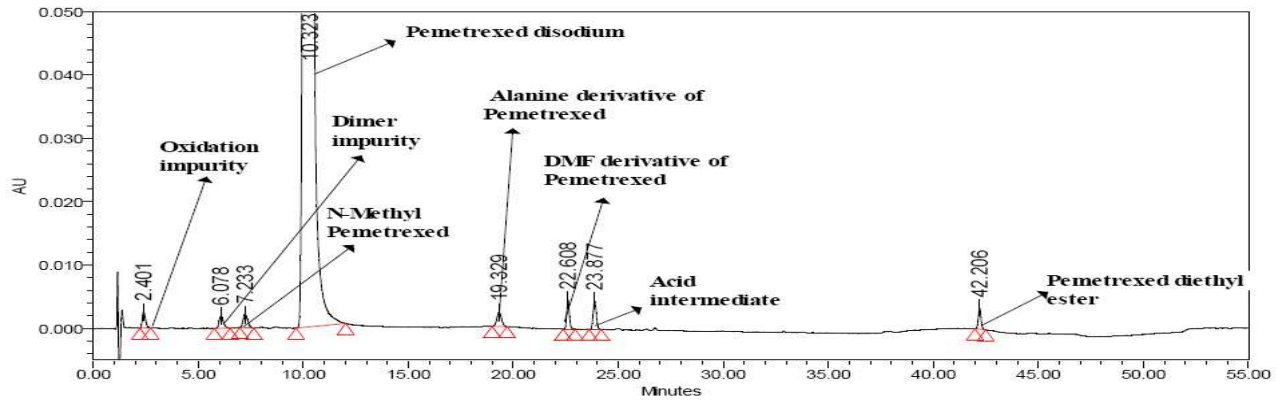
Chromatogram of Pemetrexed disodium with its process related substances



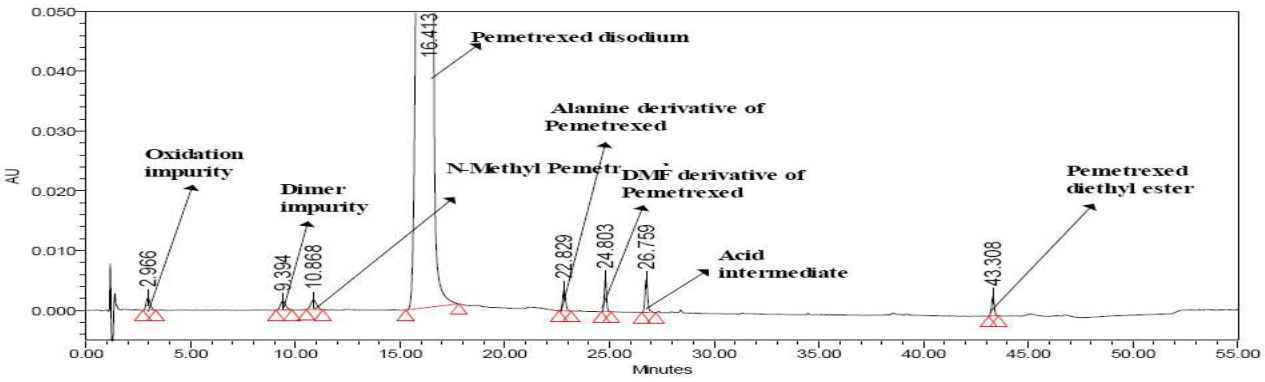
Lower flow rate (1.1 mL/min)



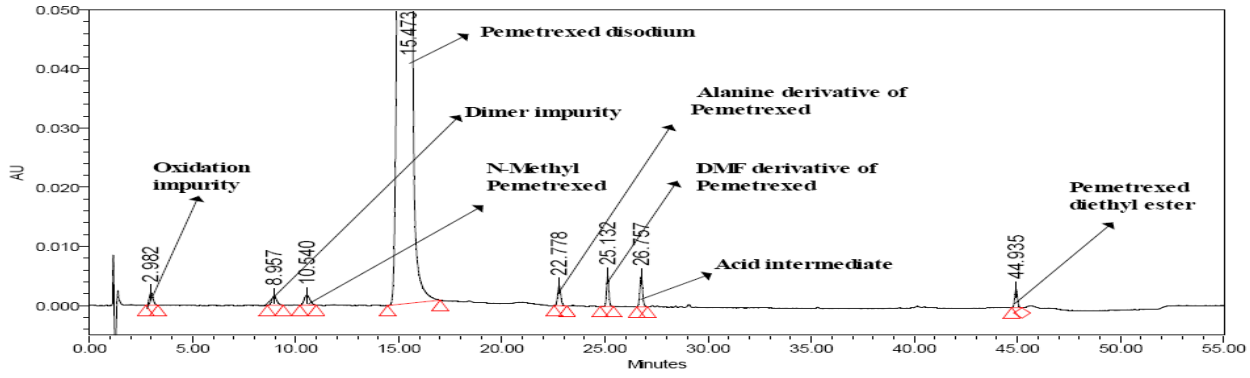
Higher flow rate (1.3 mL/min)



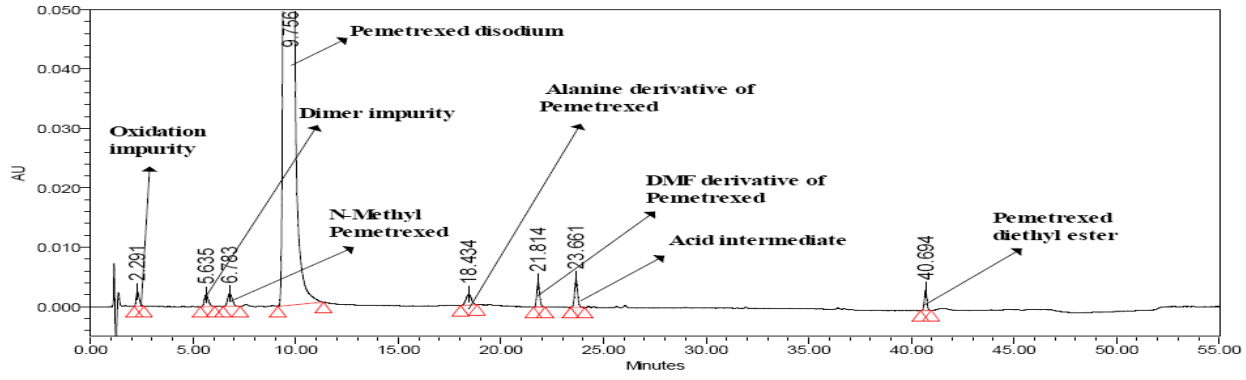
Higher column temperature (32°C)



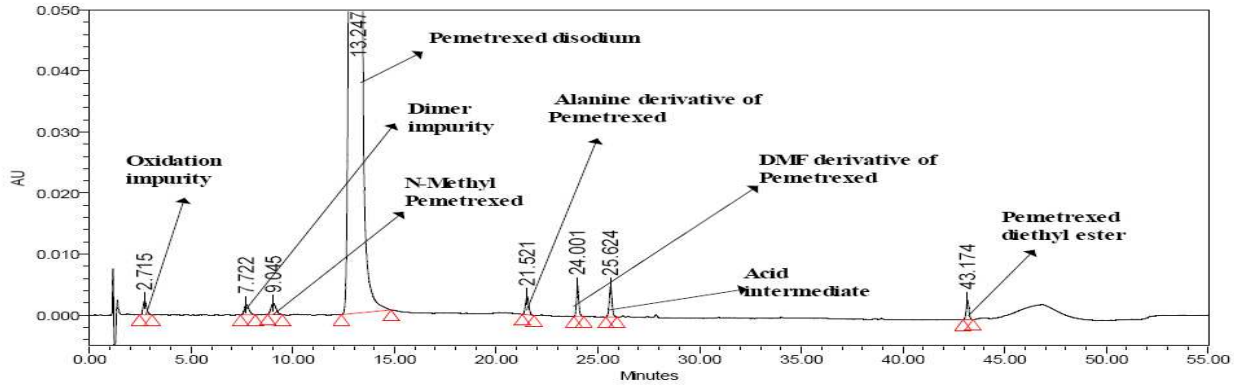
Lower column temperature (22°C)



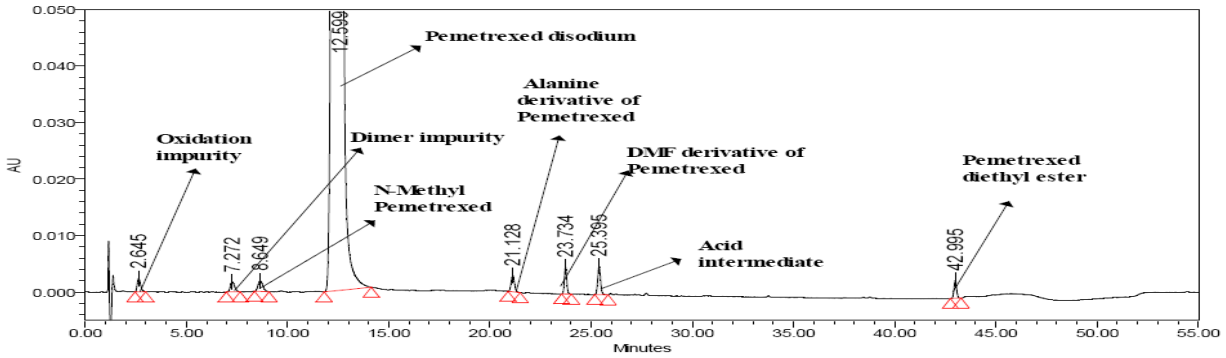
Mobile phase variation (Lower Organic)



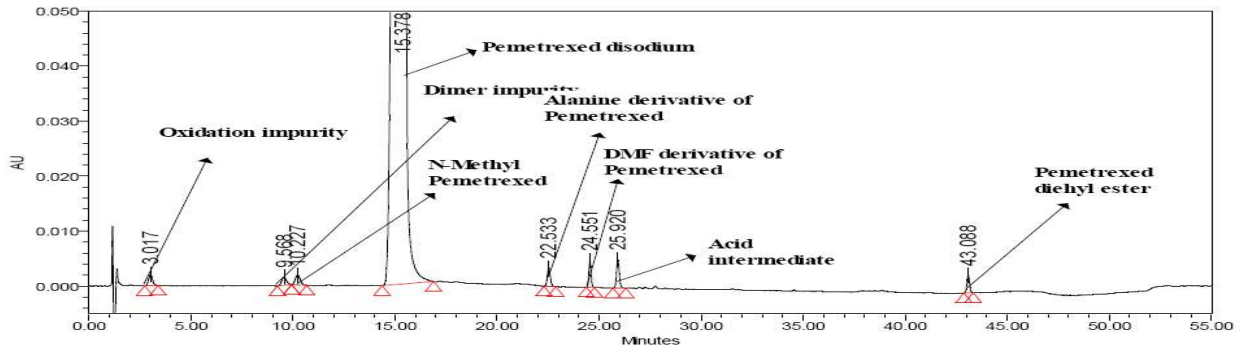
Mobile phase variation (Higher Organic)



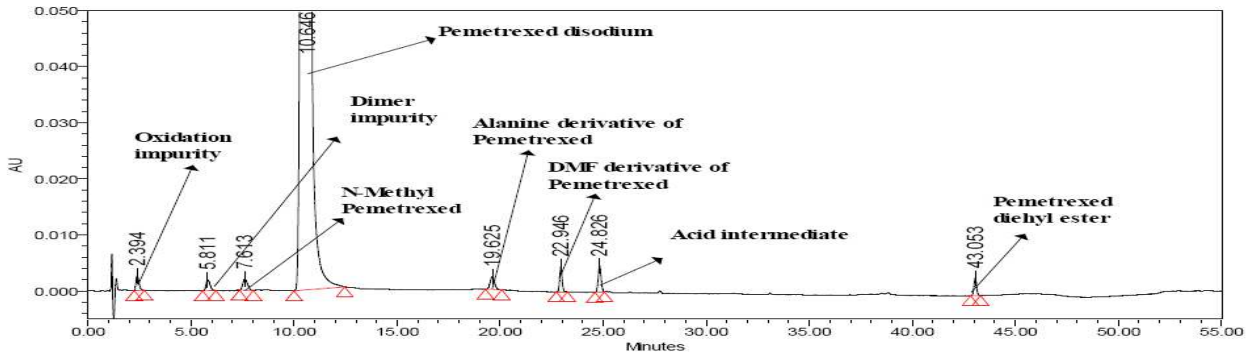
Mobile phase variation (Lower Formic acid)



Mobile phase variation (Higher Formic acid)



Buffer pH variation (pH 3.6)



Buffer pH variation (pH 4.0)

Figure 4: Robustness study of Pemetrexed disodium in presence of its process related substances

Table 9: Robustness of Pemetrexed disodium and its process related substances

Method Conditions	Pemetrexed disodium		Specificity Solution						
			Relative retention time (RRT)						
	Tailing factor	RT (mins)	Oxidation impurity	Dimer impurity	N-Methyl Pemetrexed	Alanine derivative	DMF derivative	Acid intermediate	Pemetrexed diethyl ester
As per method	0.7	12.84	0.21	0.58	0.68	1.65	1.85	1.98	3.33
Lower column temp. 22°C	0.7	16.41	0.18	0.57	0.66	1.39	1.51	1.63	2.64
Higher column temp. 32°C	0.8	10.32	0.23	0.59	0.70	1.87	2.19	2.31	4.09
Lower flow rate 1.1 mL/min.	0.7	14.00	0.21	0.58	0.68	1.57	1.74	1.86	3.10
Higher flow rate 1.3 mL/min.	0.7	11.81	0.21	0.58	0.68	1.73	1.96	2.09	3.57
Lower organic ratio (44:56,v/v)	0.7	15.47	0.19	0.58	0.68	1.47	1.62	1.73	2.90
Higher organic ratio (36:64,v/v)	0.7	9.76	0.24	0.58	0.70	1.89	2.24	2.43	4.17
Lower Formic acid ratio	0.7	13.25	0.21	0.58	0.68	1.63	1.81	1.93	3.26
Higher Formic acid ratio	0.7	12.60	0.21	0.58	0.69	1.68	1.88	2.02	3.41
Lower buffer pH-3.6	0.7	15.38	0.20	0.62	0.67	1.47	1.60	1.69	2.80
Higher buffer pH-4.0	0.8	10.65	0.23	0.55	0.72	1.84	2.16	2.33	4.04

Assay of Pemetrexed disodium

Two different brands of Pemetrexed disodium formulations were analyzed using the above optimized conditions and it was found that Pemetrexed disodium has shown 99.99-101.45 purity range and no interference of excipients was observed.

Forced degradation studies

Pemetrexed disodium was preliminarily subjected to forced degradation studies using 0.2N NaOH for 60 hours (Basic degradation), 0.2N HCl for 80 hours (Acidic degradation), 0.5% H_2O_2 for 7 hours (Oxidative degradation), UV at 254 nm for 48 hours (Photolytic degradation) and 60°C for 48 hours (Thermal degradation) with the above optimised method used for the assay method and the results were shown in Table 10. The peak purity and the purity threshold values observed during the forced degradation studies of Pemetrexed disodium conducted at an exaggerated condition were shown in Table 11 and the degradation results obtained for the related substances were shown in Table 12. The resultant chromatograms obtained during the degradation study of Pemetrexed disodium in presence of its related substances were shown in Figure 5.

Table 10: Preliminary degradation results of Pemetrexed disodium

Degradation condition	Assay (%)	Total impurities (%)	Purity angle	Purity threshold
Pemetrexed	99.2	0.99	0.062	0.201
Basic degradation (0.2N NaOH,60 h)	98.9	0.95	0.055	0.299
Acidic degradation (0.2N HCl, 80 h)	95.2	1.12	0.061	0.219
Oxidative degradation (0.5% H_2O_2, 7 h)	101.8	4.04	0.092	2.175
Photolytic degradation (UV at 254 nm, 48 h)	99.2	0.96	0.054	0.210
Thermal degradation (60°C, 48 h)	99.2	0.94	0.072	0.200

Table 11: Peak purity results of Pemetrexed disodium

Degradation condition	Purity angle	Purity threshold
Basic degradation (0.5N NaOH, 24 hrs)	0.224	0.401
Acidic degradation (0.1N HCl, 24 hrs)	0.026	0.316
Oxidative degradation (0.5% H_2O_2, 48 hrs)	0.026	0.307
Photo degradation (1.2 million Lux hours and 200 Wat Hrs/Sq.Mtr)	0.128	0.347
Thermal degradation (60°C, 9 days)	0.196	0.324

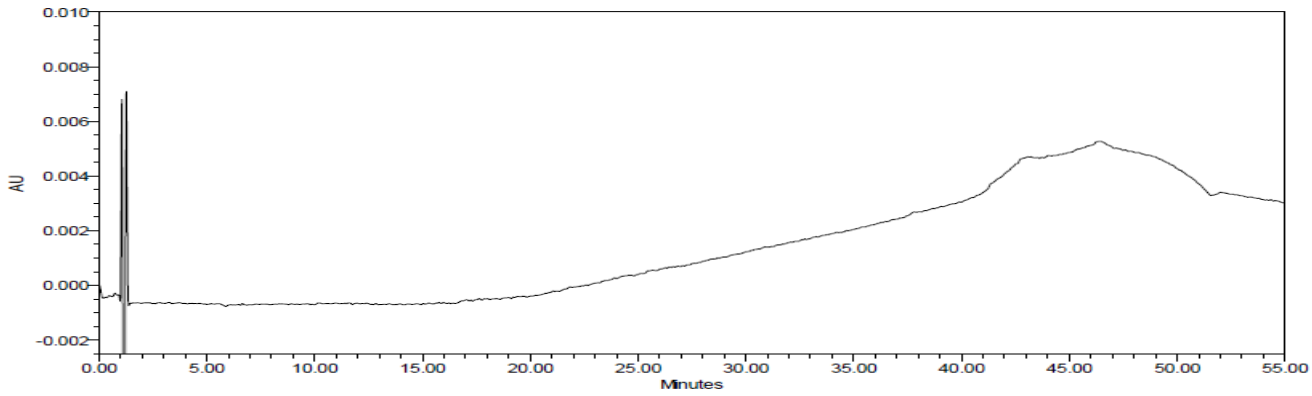


Figure 5A: Chromatogram of blank

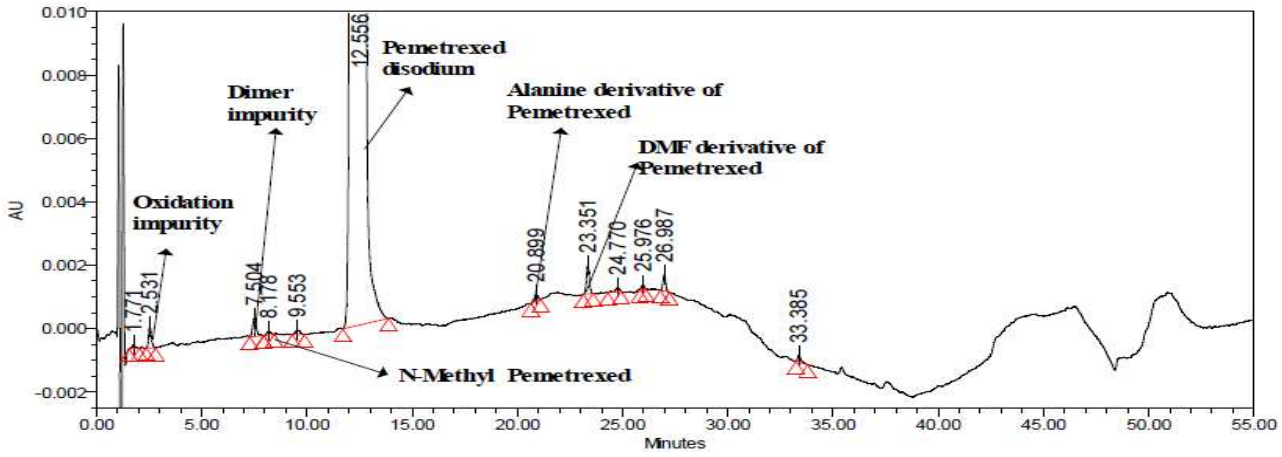


Figure 5B: Chromatogram of Pemetrexed disodium

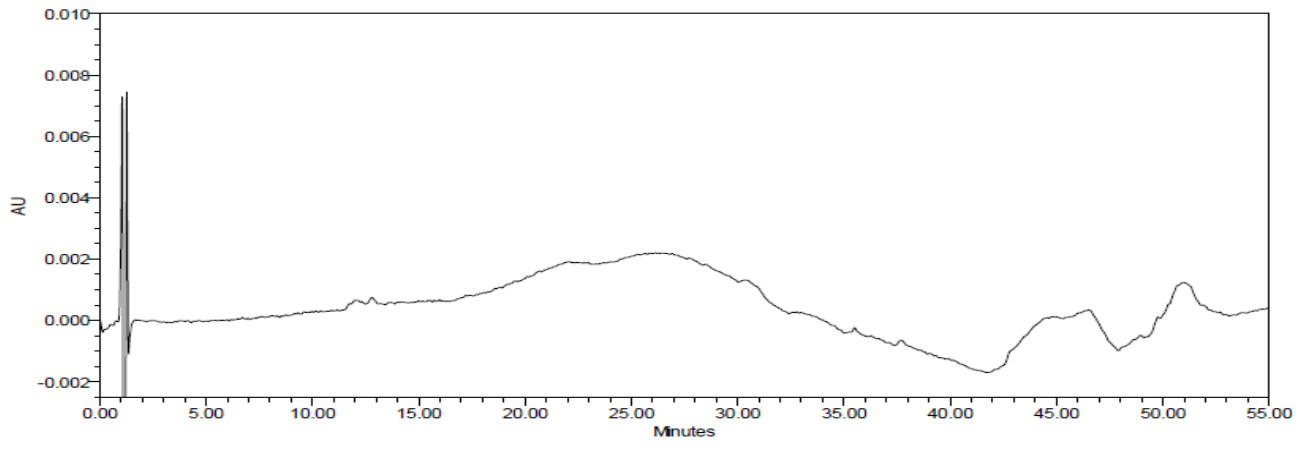


Figure 5C: Chromatogram of NaOH blank

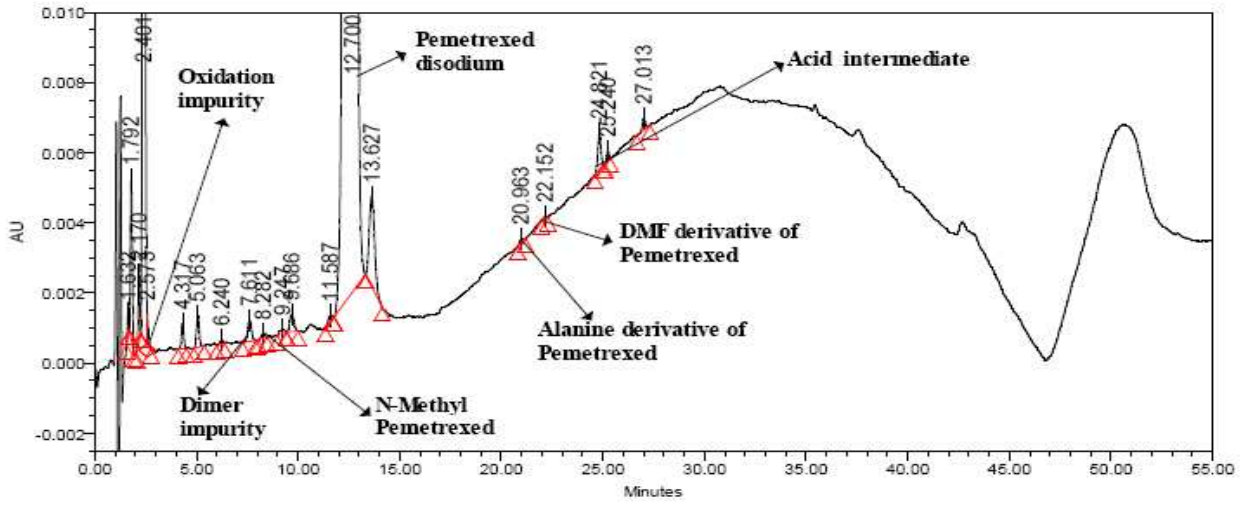


Figure 5D: Chromatogram of Pemetrexed disodium during basic degradation (0.5N NaOH)

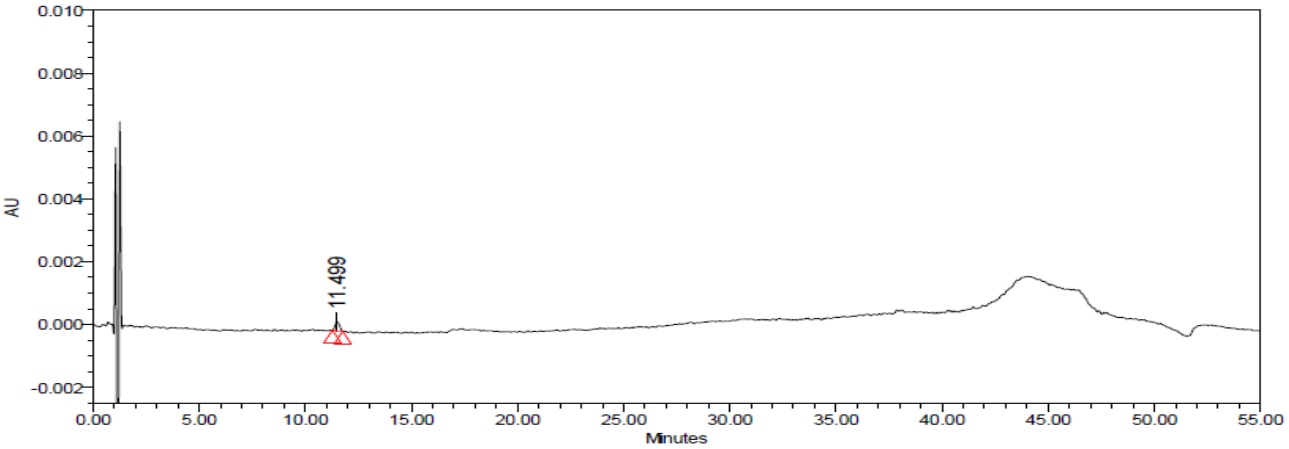


Figure 5E: Chromatogram of HCl blank

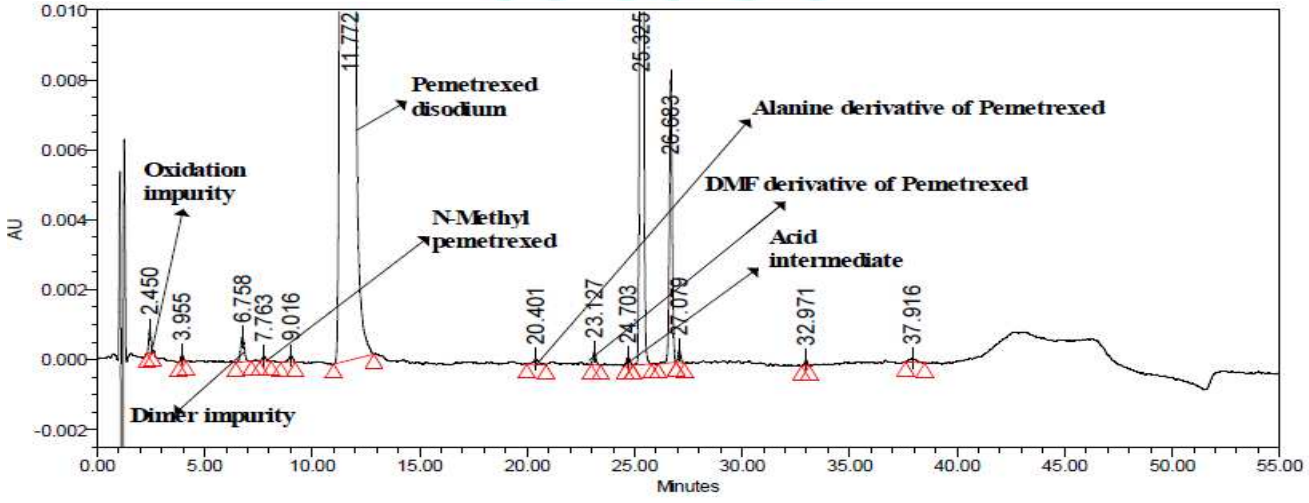


Figure 5F: Chromatogram of Pemetrexed disodium during acidic degradation (0.1N HCl)

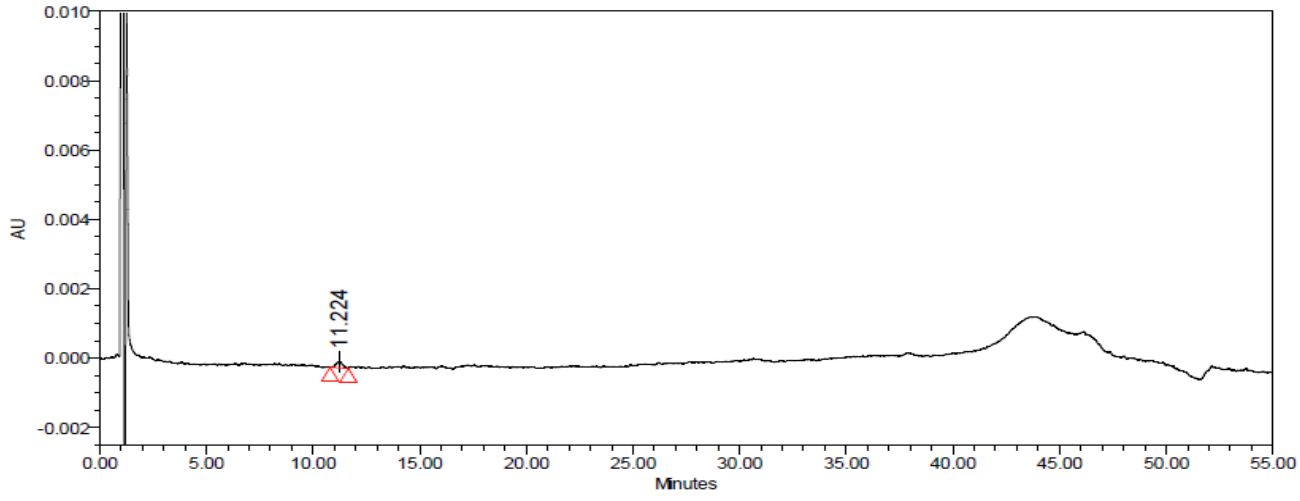


Figure 5G: Chromatogram of H_2O_2 blank

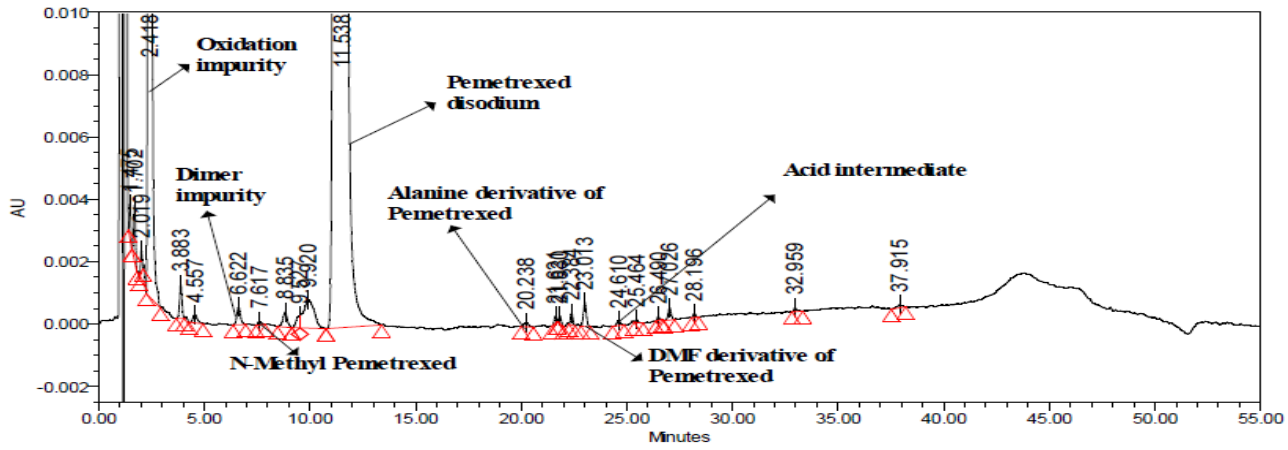


Figure 5H: Chromatogram of Pemetrexed disodium during oxidative degradation (0.5% H_2O_2)

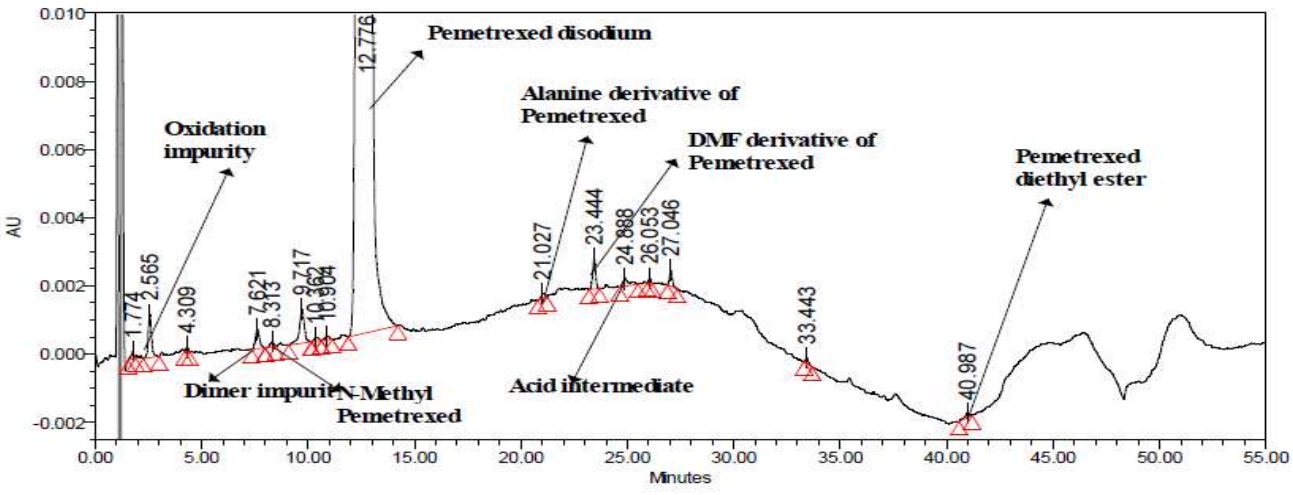


Figure 5I: Chromatogram of Pemetrexed disodium during thermal degradation

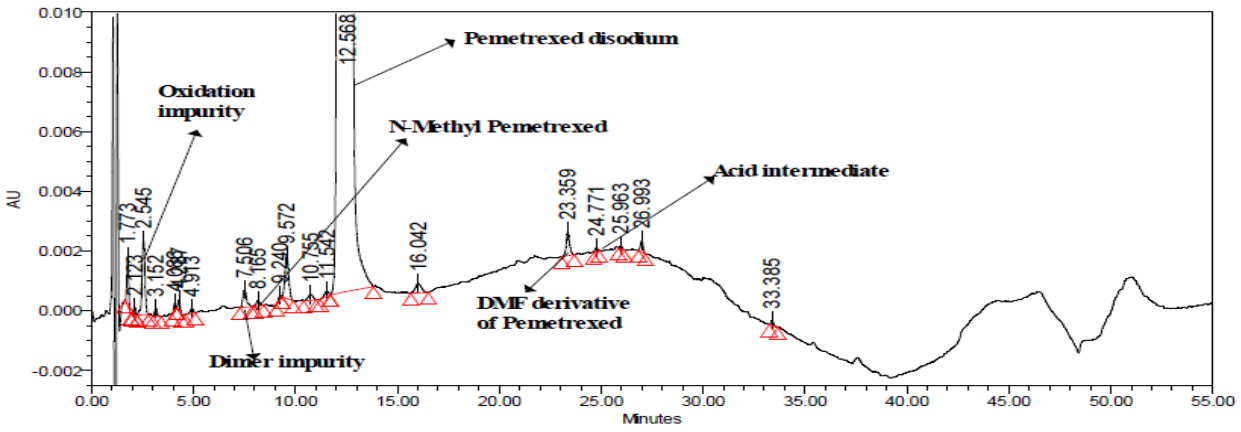


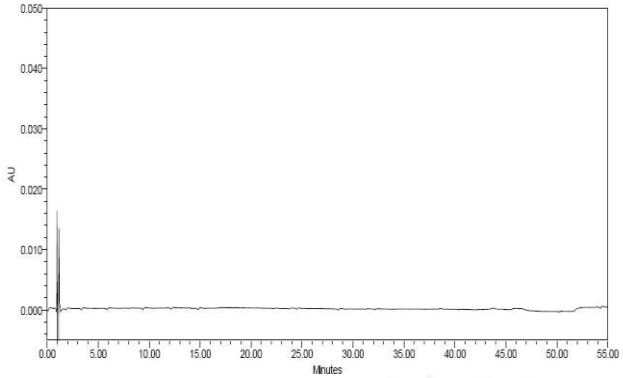
Figure 5J: Chromatogram of Pemetrexed disodium during photolytic degradation

Table 12: Degradation results of related substances of Pemetrexed

Degradation condition	% D-isomer	% w/w Oxidation impurity	% w/w Dimer impurity	% w/w N-Methyl Pemetrexed	% w/w Alanine derivative	% w/w DMF derivative	% w/w Acid intermediate	% w/w Pemetrexed diethyl ester
Degradation results of related substances during base hydrolysis (0.5N NaOH)								
Control	0.01	0.05	0.07	0.01	0.01	0.04	0.01	Below detection limit
24 hrs	0.02	0.02	0.08	0.01	0.01	Below detection limit	0.04	Below detection limit
Degradation results of related substances during acidic hydrolysis (0.1N HCl)								
Control	0.02	0.06	0.08	0.01	0.01	0.03	0.01	Below detection limit
24 hrs	0.02	0.03	0.08	0.01	0.01	0.02	0.01	Below detection limit
Control	0.02	0.06	0.08	0.01	0.01	0.03	0.01	Below detection limit
48 hrs	0.01	7.33	0.06	0.01	0.01	0.03	0.01	Below detection limit
Degradation results of related substances during oxidation (0.5% H_2O_2)								
Control	0.02	0.06	0.08	0.01	0.01	0.03	0.01	Below detection limit
48 hrs	0.01	7.33	0.06	0.01	0.01	0.03	0.01	Below detection limit
Degradation results of related substances during photolysis (1.2 million lux hours and 200 Wat Hrs /Sq.Mtr)								
Control	0.01	0.05	0.07	0.01	0.01	0.04	0.01	Below detection limit
After 1.2 million lux hours	0.02	0.19	0.07	0.01	Below detection limit	0.04	Below detection limit	Below detection limit
Thermal degradation results of related substances (60°C, 9 days)								
Control	0.01	0.05	0.07	0.01	0.01	0.04	0.01	Below detection limit
9 days	0.02	0.10	0.07	0.01	0.01	0.04	0.02	0.01

Specificity

Specificity of the method was determined by injecting the analyte spiked with all the known components expected to be present in the drug substance. Separate solutions of diluent, Pemetrexed disodium, known impurities (Oxidation impurity, Dimer impurity, Pemetrexed diethyl ester, DMF derivative of Pemetrexed, Alanine derivative of Pemetrexed, Acid intermediate and N-Methyl Pemetrexed) and a combined solution containing all the mentioned components were injected at 0.15% for Oxidation impurity, Dimer impurity, Pemetrexed diethyl ester, DMF derivative of Pemetrexed, Alanine derivative of Pemetrexed, Acid intermediate and N-Methyl Pemetrexed into a HPLC. The peak of any substance in the given optimized conditions is considered to be spectrally pure if the purity angle is less than he purity threshold. Resolution between Pemetrexed disodium peak and nearby peak was not less than 1.5. Resolution obtained between Pemetrexed disodium and nearby peak is 4.6. Table 13 shows the retention time, relative retention time (RRT), relative response factor (RRF) and peak purities w.r.t. Pemetrexed disodium in the combined solution.



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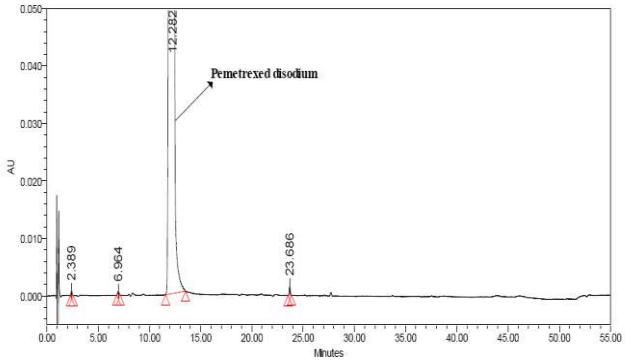
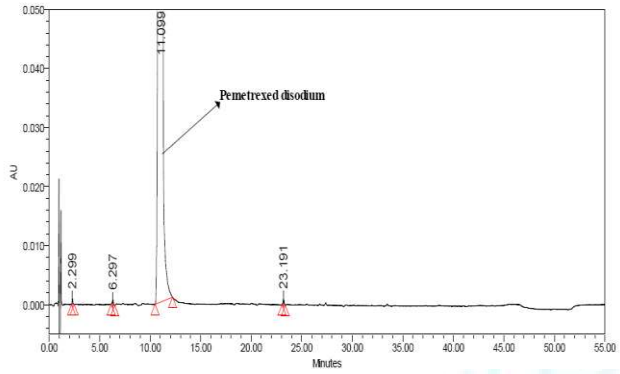
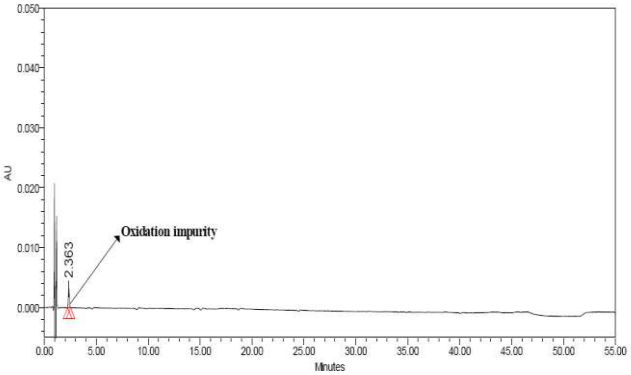


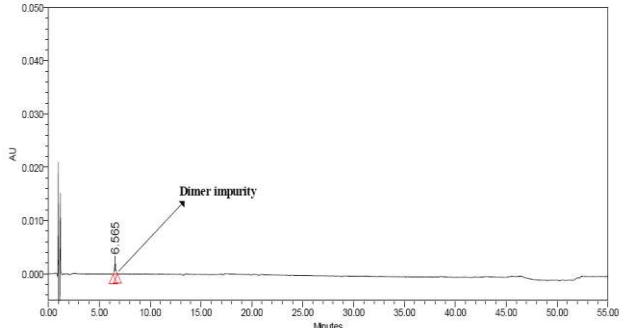
Pemetrexed disodium standard



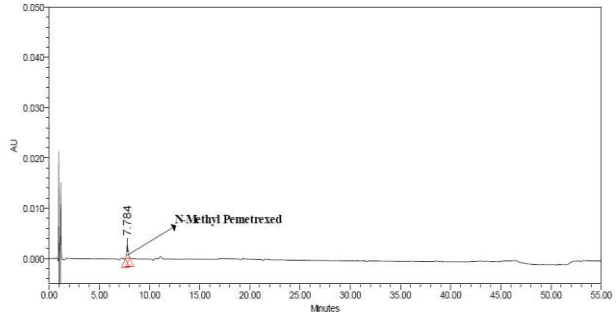
Pemetrexed disodium (As such solution)



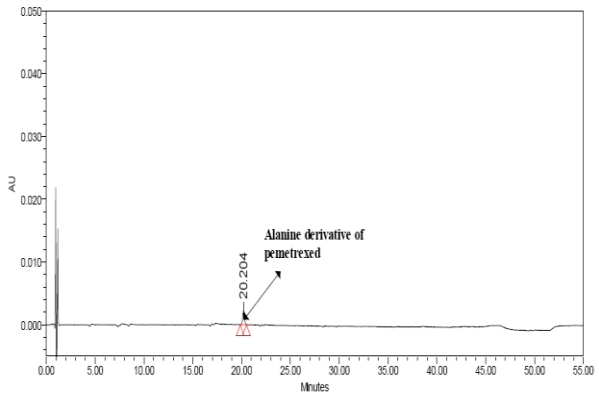
Oxidation impurity Standard Solution



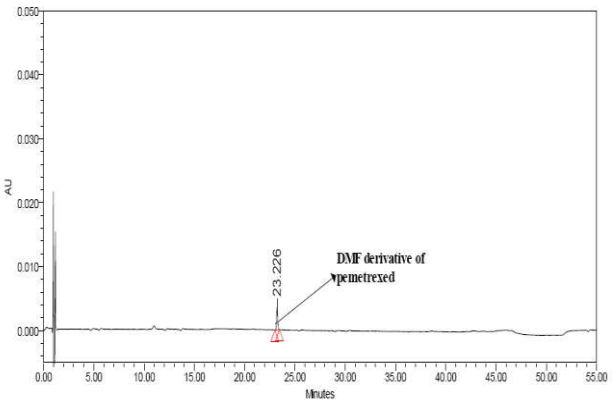
Dimer impurity Standard Solution



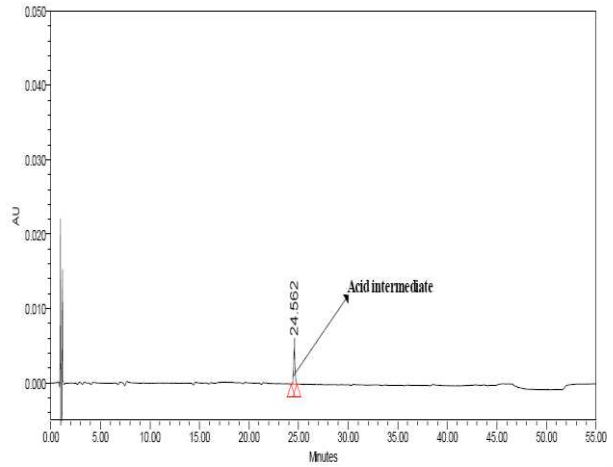
N-Methyl Pemetrexed Standard



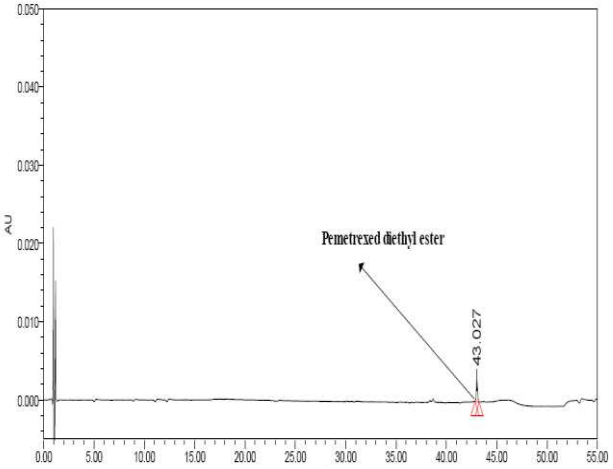
Alanine derivative of Pemetrexed Standard



DMF derivative of Pemetrexed Standard



Acid intermediate Standard



Pemetrexed diethyl ester Standard

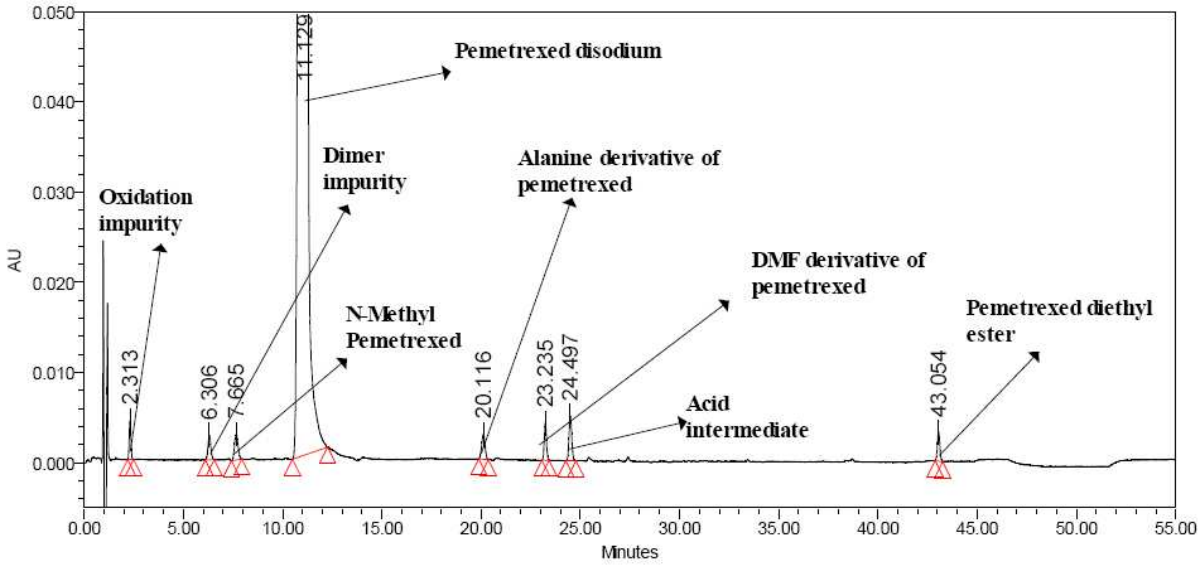


Figure 6: Chromatograms of Pemetrexed disodium and its process related substances (Specificity)

Table 13: Specificity

Component	RT (min)	Relative retention time (RRT)	Relative response factor (RRF)	Resolution	Purity angle	Purity threshold
Pemetrexed disodium	14.57	1.00	---	2.1	0.020	0.244
Oxidation impurity	2.88	0.20	1.45	18.8	0.729	2.797
Dimer-1 impurity	7.78	0.52	1.85	4.6	0.848	2.279
N-Methyl Pemetrexed	11.24	0.78	1.02	4.6	1.886	2.425
Dimer-2 impurity	16.57	1.14	1.14	2.1	0.214	0.564
Alanine derivative of Pemetrexed	22.50	1.54	0.95	2.2	1.742	2.202
DMF derivative of Pemetrexed	24.99	1.72	0.91	5.4	0.717	2.000
Acid intermediate	27.09	1.86	0.72	2.6	0.492	2.222
Pemetrexed diethyl ester	44.64	2.06	1.12	2.4	0.798	2.981

DISCUSSION

Pemetrexed disodium and its process related substances [N-Methyl Pemetrexed, Pemetrexed diethyl ester, Alanine derivative of Pemetrexed, Dimer-1 impurity, Dimer-2 impurity, DMF derivative of Pemetrexed, Acid intermediate, Oxidation impurity and D-isomer] were separated on gradient mode and quantified using using liquid chromatographic technique. This method has not been done till today in the literature and a brief summary of the analytical methods so far developed by authors was given in Table 14. (0.02M sodium dihydrogen phosphate with 0.1% HCOOH and pH 3.8 with dilute sodium hydroxide): Acetonitrile (40:60 v/v). The system suitability, specificity and other validation parameters were well in accordance with the ICH guidelines.

Table 14: Comparison of published methods with the present method

Method	Mobile phase (v/v)	Comment	Ref
Ultrafast and high-throughput MALDI-QqQ-MS/MS analysis Methotrexate internal standard	-	Human plasma	4
LC–MS/MS analysis Isotope-labelled internal standard	-	Human plasma Pemetrexed and its metabolites	5
HPLC Internal standard Lometrexol	sodium formate buffer: acetonitrile	Human plasma and urine	6
UPLC	0.1% ortho-phosphoric acid: Acetonitrile	Stability indicating	7
Chiral HPLC	Hexane: Ethanol: Trifluoro acetic acid	Separation of D and L-enantiomers	8
Chiral HPLC	Hexane: Ethanol: Isopropyl alcohol: TFA (250:650:100:1)	Separation and assay of Pemetrexed and its D isomer	9
HPLC	0.1% v/v aq. phosphoric acid buffer: acetonitrile (85: 15)	Stability indicating assay	10
HPLC	Phosphate buffer (pH adjusted to 6.5 with ortho phosphoric acid): acetonitrile (90: 10)	Assay	11
HPLC	Acetonitrile: Phosphate buffer (pH adjusted to 3.0 with ortho phosphoric acid): (35: 65)	Assay	12
HPLC	Acetonitrile and buffer (pH adjusted to 5 with orthophosphoric acid) in the ratio (15:85)	Assay and its related substances	13
HPLC Spectrophotometry	20 mM Dibasic phosphate buffer (adjusted to pH 6.50 with ortho-phosphoric acid) and acetonitrile (88:12) Distilled water	Assay	14
Spectrophotometry	1,2-Napthoquinone-4-Sulphonic acid MBTH reagent PDAB reagent	Assay	15
Voltammetry technique	Phosphate buffer pH 3 - pH 10	Linearity 10 µM to 0.75 µM	16
HPLC (Gradient mode)	Buffer [0.02M sodium dihydrogen phosphate with 0.1% HCOOH (pH adjusted to 3.8 with dil. NaOH)] : [Buffer: Acetonitrile (40:60)	Linearity 0.5-1500 µg/mL	Present method

CONCLUSIONS

A simple and new stability indicating RP-HPLC method has been developed for the determination of Pemetrexed disodium and its related substances. The method was validated (ICH guidelines) by linearity, precision, accuracy and robustness and this method is highly helpful for the identification and quantification of impurities and related substances in injections as well as metabolic studies. The proposed method is specific and the system suitability parameters are within acceptable criteria.

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