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A new rapid Stability indicating RP-PDA-UPLC method for the estimation of Assay of Pemetrexed disodium-An anti-Lung cancer drug from lyophilized parenteral formulation

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ABSTRACT

A new rapid stability-indicating reversed phase ultra-performance liquid chromatographic (RP-UPLC) method with a linear gradient and shortest run time of 4.0 minutes is developed for the determination of assay of pemetrexed disodium, an anti-cancer drug from its lyophilized parenteral formulation. The method is developed on Waters binary UPLC, equipped with Aquity BEH C18 column and system set with mobile phase A as 0.1% *ortho*-phosphoric acid and B as Acetonitrile. The drug product is exposed to forced stress conditions like peroxide, acid, base, hydrolytic, thermal, and photolytic degradation, within which major degradants were observed in acid stress at 1N HCl 60°C and 3% peroxide stress at room temperature. Pemetrexed (main peak) and its degradant peaks were well separated and were monitored at UV-230nm. Evaluation of spectral purity for main component is performed using PDA (photo diode array) in presence of its degradants formed during stress studies, which assures the stability indicating capability of the method. % RSD for mean of precision and accuracy at 3 different levels ranging from 50 to 150% were within limits and coefficient of correlation found > 0.999 for linearity. The newly developed UPLC assay method is fully validated and found to be specific, Robust, Precise, Linear and Accurate in determining assay of Pemetrexed from drug product as per ICH guidelines.

INTRODUCTION

Non-small cell lung cancer (NSCLC) accounts for about 85% of all lung cancers cases and is considered as leading cause of cancer death in the United States and worldwide. It is also sub classified as squamous (~30%) or non-squamous (~70%) which includes adeno carcinoma and large cell histologies types. NSCLC is a particularly aggressive form of lung cancer, for which there is a lack of effective and well-tolerated treatments available. (Julian *et al*., 2008) Although new cytotoxic agents and targeted therapies were evaluated, but their efficacy is limited for first-line therapy of NSCLC. Based on recent advances in clinical trials and results of the phase III studies, pemetrexed an multi-targeted antifolate drug progressively became one of the most frequently used cytotoxic chemotherapy agents for treating stage IV nonsquamous NSCLC (Hanauske *et al*., 2001, Pascale *et al*., 2016). Pemetrexed is approved by the Food and Drug Association (FDA) for several steps of nonsquamous NSCLC treatment (first line, maintenance therapy, and second and third lines). Pemetrexed is indicated as a single-agent for the treatment of patients with locally advanced or metastatic nonsquamous non-small cell lung cancer after prior chemotherapy. Pemetrexed, in combination with cisplatin is also indicated for the treatment of patients with malignant pleural mesothelioma whose disease is unresectable or who are otherwise not candidates for curative surgery (Adjei *et al*., 2003). Pemetrexed disodium heptahydrate has the chemical name L-Glutamic acid,N-[4-[2 -(2-amino-4,7-dihydro-4-oxo1H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]benzoyl]-, disodium salt, heptahydrate with a molecular formula of $C_{20}H_{19}N_5Na_2O_6$ $7H_2O$ and a molecular weight of 597.49 amu (Figure-1).

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The drug product is supplied as a sterile lyophilized powder for intravenous infusion available in single-dose vials in two strengths of 100-mg and 500-mg along with mannitol at approximately 1:1 ratio. (http://www.rxlist.com/alimta-drug.htm [Accessed on 02-03-2017].

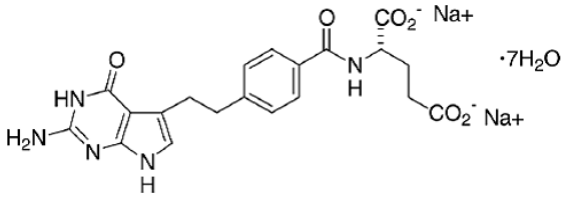

Fig. 1: Chemical Structure of Pemetrexed Disodium.

According to literature survey, few methods were reported about evaluation of assay of pemetrexed and its stability in solutions by HPLC (Saravanan *et al*., 2007), (Banu *et al*., 2010), (Rondelot *et al*., 2007), (Zhang *et al*., 2006). Few methods describe about LC-MS studies of pemetrexed and its metabolites in biological fluids like plasma and urine (Roland *et al*., 2010), (Meesters *et al*., 2010), (Li Wang *et al*., 2006) (Rivory *et al*., 2001). Recently, UPLC which works on sub-2µ particle size packed columns became a novel technique in industrial research and quality control sectors, ensuring faster and adequate analytical output without compromising quality of analysis, eventually improving productivity (Mazzeo *et al*., 2005, Wren *et al*., 2006, Nguyen *et al*., 2006, Villiers *et al*., 2006, Russo *et al*., 2008). Over past few years, analytical research works and publications on UPLC were tremendously increasing, and hence the technique is chosen for current work. As per literature survey, no RP-UPLC assay method is available with shortest run time, which established stress studies of Pemetrexed drug product along with spectral purity (PDA) analysis with negligible carry over. Analytical procedure for assay for this molecule is currently not listed in any official pharmacopeia and this will be first of kind on UPLC. The newly developed method is fully validated in terms of specificity, precision, accuracy, linearity, ruggedness and robustness as per ICH and regulatory guidelines (Q2 (R1), 2005).

MATERIALS AND METHODS

Chemicals and reagents

HPLC gradient grade (0.2µ Filtered) acetonitrile, HPLC grade water and *ortho*-phosphoric acid (85%) is procured from Thermo Fisher suppliers, Hyderabad. Pemetrexed disodium standard is received as research sample from Department of Biochemistry, Acharya Nagarjuna University, Guntur, Andhra Pradesh. Drug product (Strength 100mg/vial) is purchased from MDC-NIMS, Hyderabad. Mannitol, as a placebo constituent is procured from alfa aesar Ltd, Hyderabad.

Equipments

Chromatographic conditions of the developed method is optimized on Acquity UPLC™ binary system (Waters, Milford, USA) equipped with a sample manager and a photodiode array (PDA) detector. Chromatograms and spectral data is monitored, integrated and processed using empower-2 software. Photo stability chamber (Sanyo, Leicestershire, UK), Dry air oven (Cintex, Mumbai, India), Digital water bath (Thermo Scientifics, USA), were used to achieve respective photolytic, thermal, acid and alkali stress conditions. Ultra sonication (Power sonic 420, Labtech, Korea) is utilized during degassing of Mobile phases and preparation of Solutions.

Chromatographic Conditions

Refer Table-1 for chromatographic conditions implemented for the current study. 0.2 µm membrane filtered HPLC water is used during mobile phase preparation. Sample manager is set with 10 µL loop and characterized seal and loop volume before start.

Pemetrexed and its degradants peaks were monitored at UV-wavelength of 230 nm, with sampling rate of 10 point/sec, 1.2 nm resolution.

Table 1: UPLC chromatographic conditions.

Column	Aquity BEH C18, 100 x 2.1 mm, 1.7µm		
Mobile Phase : A	0.1% *Ortho*- Phosphoric Acid in Water		
Mobile Phase : B	Acetonitrile		
Flow rate	0.3 mL/min		
Column oven temperature	40°C		
Sample temperature	25°C		
Injection Volume	4 µL		
Strong needle wash	10:90v/v Water: acetonitrile, 500µL		
Weak needle wash	90:10v/v Water: acetonitrile, 500µL		
Linear Gradient Program			
Time (Min)	% Solvent- A	% Solvent- B	Curve
0.0	85	15	-
1.5	70	30	6
2.4	50	50	6
2.5	85	15	6
4.0	85	15	6

Preparation of Standard and Sample Solutions

0.22µ filtered HPLC grade water is used as diluent. Standard stock solution of pemetrexed is prepared by dissolving 40 mg of working standard in diluent followed by further dilutions to meet 40µg/mL. Decrimped 5 vials containing lyophilized content of label claim equivalent to 100mg of pemetrexed, added 5 mL diluent to the vial. Wet the content, close with lid and completely dissolve the content by thorough vortexing and transfer the solution to 250 mL volumetric flask. Repeatedly rinse the vials 3 times with diluent and transfer the rinsing to the flask and make up 80% of final volume with diluent and thoroughly vortex for few minutes.

Dilute rest of volume with diluent to meet 0.4 mg/mL (Test stock). Final test concentration of 40 µg/mL is attained by diluting test stock with diluent.

METHOD VALIDATION

System Suitability and Precision

System suitability is checked by injecting 5 repeated injections of standard solution. USP tailing factor (General chapter 611) of below 1.5, % RSD of < 2% were obtained. A series of six individual samples were prepared and Repeatability (Precision) of the method is assessed by evaluating % assay and %RSD within acceptance criteria. The intermediate precision (Intra-Day) of the assay method is evaluated by different analyst on different day.

Accuracy and Linearity

The Accuracy of the assay method is evaluated using three concentration 50%, 100%, 150% to target test concentration and % added, % found and % recovered were evaluated. Keeping placebo weight constant, pemetrexed API is spiked at specified levels to attain range and to evaluate extraction efficiency. To establish Linearity of the assay method, a series of solutions at five concentration levels ranging from 25 to 200% of final test concentration (10 to 80 µg/mL) were prepared from standard stock solution. The peak area versus concentration (in µg/mL) data for main peak (pemetrexed) is subjected to least-squares fit linear regression analysis and Slope, Y-Intercept, coefficient of correlation and bias at 100% response were evaluated.

Specificity-Forced Degradation

Specificity is the ability of an analytical method to measure the analyte response accurately in the presence of its potential impurities (Q2 (R1), 2005), which may be degradants or process related. The study also emphasizes the separation efficiency of the developed methods from placebo and other extraneous peaks, and evaluate the impact on quantification of peak of interest. In current study, the drug product is subjected for intentional degradation and is exposed for physical stress condition like thermal, humidity and photolysis. Test solution (40 µg/mL) were degraded by applying chemical stress conditions like acid, alkali and peroxide. Finalized stress conditions were applied for placebo and treated as same as stressed samples. Prior to loading in UPLC system for analysis, respective stressed samples were allowed to attain room temperature and subjected for neutralization. Refer to Table-3 for forced degradation details and stress conditions.

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered to evaluate the impact of change in chromatographic conditions (Flow rate and column temperature). A change in flow rate of ±10% i.e., 0.45 mL/min and 0.55 mL/min from actual flow rate and a change in ± 5º C i.e., 35º C and 45º C of actual column temperature were studied to evaluate impact on system suitability.

Solution Stability and Mobile Phase Stability

The stability of solutions is determined by leaving solutions of the sample and standard in tightly capped volumetric flasks at room temperature from the day of preparation till 48 hours. The mobile phase prepared at the beginning of the study and set on UPLC system, is unaltered during the experiment and subsequently tested at an interval of 48 hours. Stability of mobile phase and solutions were determined by comparing with freshly prepared standard and difference in % assay is calculated.

RESULTS AND DISCUSSION

Method development Optimization

As the pKa of pemetrexed is 3.34 (Strongest Acidic), and UV absorbance maxima at 230nm, Ortho phosphoric acid along with acetonitrile is chosen as mobile phase constituents (UV cut off <205nm). Pemetrexed, being disodium salt and formulated with mannitol is freely soluble in water, and hence used as diluent for extraction. Initial trials were performed starting with a short linear gradient with Aquity 50 x 2.1 mm, 1.7µ column and checked for reproducibility. Symmetric peak shape is not obtained due to early elution of peak and henceAquity100 x 2.1mm 1.7µ column is selected and trails were performed to separate degradants generated during stress studies. With a linear gradient Time (in minutes)/%B: 0.00/20, 1.20/40, 3.20/60, 3.80/80, 3.90/20, 5.00/20, at flow rate of 0.5 mL/min, USP resolution of <1.9 is obtained between main peak and major acid degradant. With flow rate of 0.3 mL/min, Time (in minutes)/%B: 0.00/15, 1.50/30, 2.40/50, 2.50/15, 4.00/15, satisfactory separation is achieved for main peak from acid and Oxidative degradant peaks with column temperature at 40ºC. With 3 µL injection volume, symmetric peak shape with tailing factor of <1.3 and suitable area count is obtained for main peak at a concentration of 40 µg/mL in both standard and sample solutions. During analysis, carry over for main peak is noticed, which is rectified by using mix of 90:10 v/v Water: ACN as weak needle, set at 500 µL and mix of 90:10 ACN: Water v/v as Strong needle, set at 500 µL.

Specificity-Forced degradation

All forced stressed samples were analyzed as per UPLC conditions (Section 2.2 and 2.3) using PDA detector to ensure spectral homogeneity and peak purity. Potential degradation of pemetrexed is observed in acid, peroxide stress conditions. >10% degradation is achieved in acid stress, where single major degradant at RT 2.134 min is noticed. >15% degradation is achieved in peroxide stress, where two major degradants were noticed at RT 1.015 and 1.331 min. Although 5% degradation is achieved in alkali stress, no impact on peak purity is observed. No significant degradation is obtained with rest of the stress conditions, like thermal, photolytic and water hydrolysis. Blank and stressed placebo doesn't show any interference at retention time of main peak. Gradient is extended for 5 minutes to wash out any degradants (if any), and all peaks were eluted below 2.5 minutes.

Table 2A: System suitability results.

System suitability	Retention time in min (n=5)*	USP Tailing (n=5)*	% RSD (n=5)*
Method Precision	1.55	1.24	0.6
Inter-Precision	1.52	1.23	0.4

Table 2B: Precision results.

Method Precision	**Sample-1**	**Sample-2**	**Sample-3**	**Sample-4**	**Sample-5**	**Sample-6**	**Mean ****	**% RSD****
%Assay	101.0	100.9	100.5	100.3	100.7	100.4	100.6	0.28
Inter-Precision	**Sample-1**	**Sample-2**	**Sample-3**	**Sample-4**	**Sample-5**	**Sample-6**	**Mean**	**% RSD**
%Assay	100.1	99.7	101.1	100.9	99.9	100.3	100.3	0.56

*For Standard, ** Acceptance Limits: Assay = 97% to 103% for n=6 test preparations and % RSD ≤ 2.0

Solution Stability: For Standard-Similarity Factor: New vs 48 Hours Standard: 1.01 (limit: 0.98 to 1.02).
For sample % Difference: Initial vs 48 Hours Sample: 0.51 (Limit: ± 2.0%).

Mobile Phase Stability: After 48 Hours : % RSD for standard (n=5): 0.7, USP Tailing: 1.22,

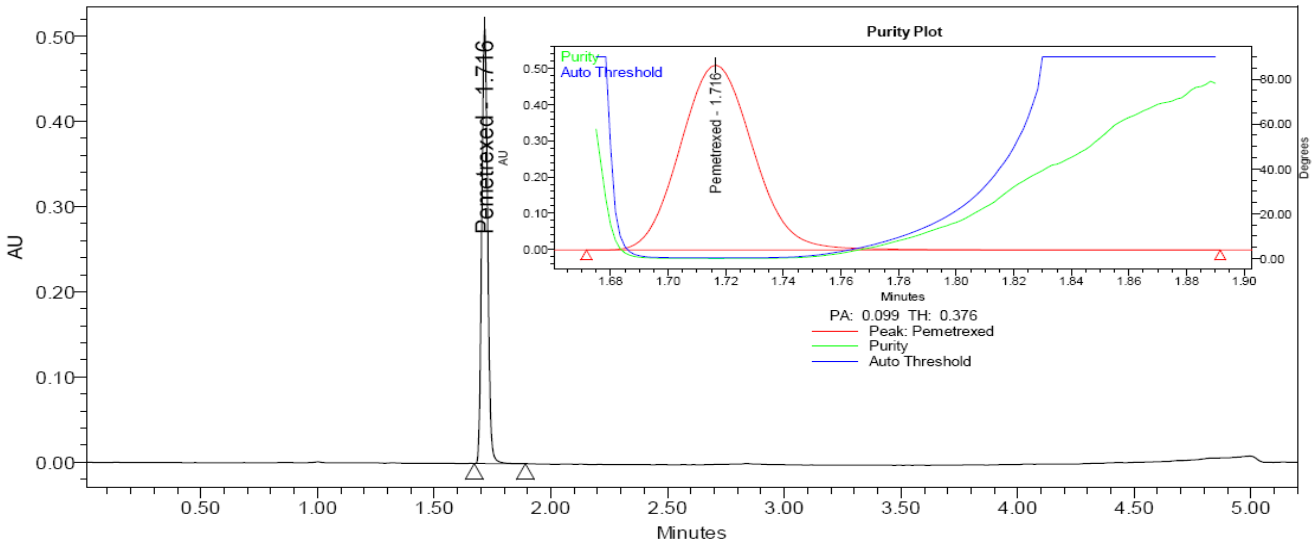

Fig. 2A: Typical Chromatogram of Unstressed Sample.

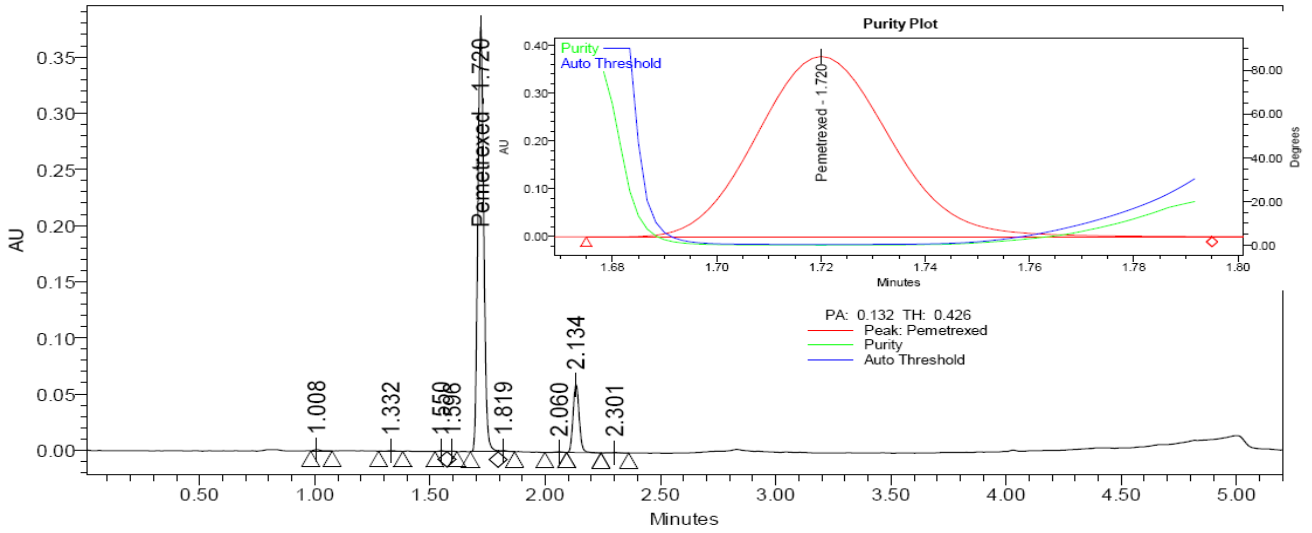

Fig. 2B: Typical Chromatogram of Acid Stressed sample.

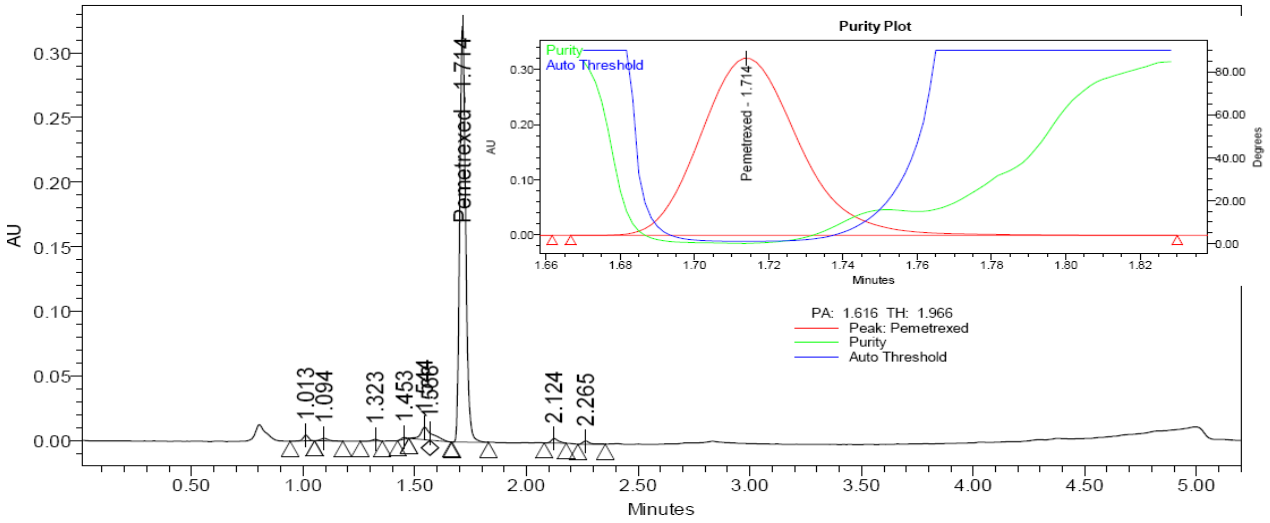

Fig. 2C: Typical Chromatogram of Alkali Stressed sample.

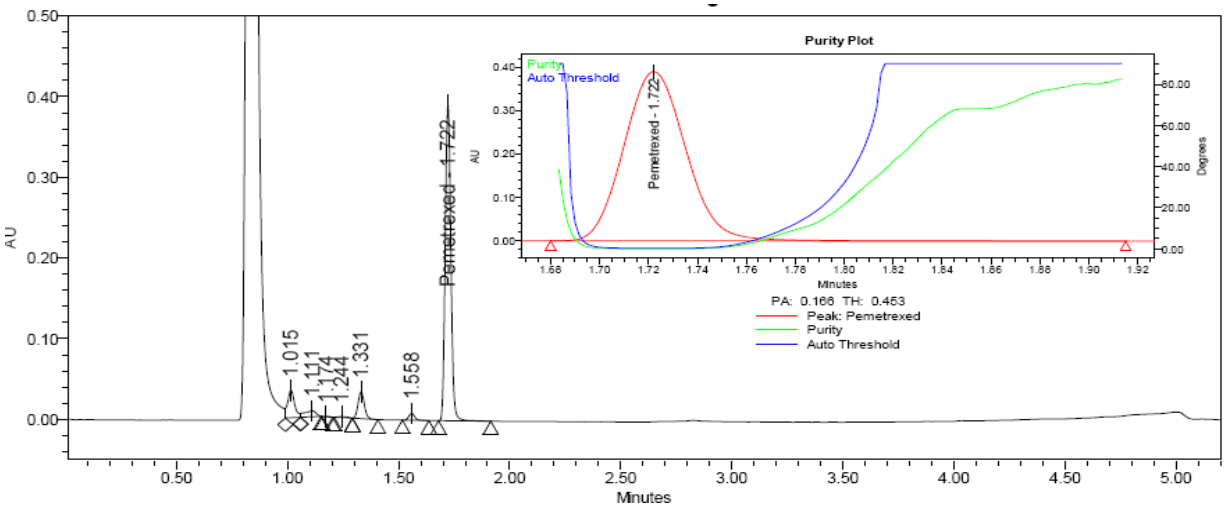

Fig. 2D: Typical Chromatogram of Peroxide Stressed sample.

Table 3: Results of Stress Studies.

Stress Condition	Stress Conditions	% Degraded**	Purity Angle*	Purity Threshold*	Purity Flag*	Purity Error*
Unstressed Sample	Not applicable	0.00	0.099	0.376	No	No
Acid Stressed Sample	1N HCl, 60°C in water bath, 3 hours	15.32	0.132	0.426	No	No
Alkali Stressed Sample	1N NaOH, 60°C in water bath, 3 hours	5.93	1.616	1.966	No	No
Peroxide Stressed Sample	3% H_2O_2 30 min, Room temperature	17.15	0.166	0.453	No	No
Humidity Stressed Sample	90%RH/25°C Saturated KNO_3	0.18	0.191	0.463	No	No
Photo Stressed Sample	1.2 M Lux hours-ICH	0.21	0.107	0.398	No	No
Hydrolysis Stressed Sample	Water, 60°C in water bath, 3 hours	1.76	0.109	0.370	No	No
Thermal Stressed Sample	105°C for 24hours	0.00	0.100	0.382	No	No

(*) For peak purity as per Waters Empower software: Purity angle must be less than purity threshold, and must have no purity flag and purity error.
(**) % Degraded = % assay of unstressed sample - % Assay of stressed sample.

All samples were screened by PDA from UV 200 to 400 nm, where no extra peaks were noticed. Peak purity is assessed by ensuring peak purity <purity threshold with no purity flag for all stressed and unstressed samples. Spectral purity of the peak and no impact of assay of pemetrexed under the influence of optimized stress conditions and potential degradants, confirm the stability-indicating capability of the newly developed method. Refer Table-3 and figure 2A to 2D for chromatograms of stress studies.

System suitability and Precision

System suitability parameter is passed and is within acceptable limits. The % RSD of assay during the method's precision and intra-day study is 0.28 and 0.56 and results found reproducible, hence forth conforming the suitability and repeatability of the method. Refer Table-2A and 2B.

Accuracy and Linearity

The mean recovery of pemetrexed at three levels 50%, 100% and 150% is ranged from 99.89% to 100.76% with a %RSD below 2% per triplicate sample preparation for each level. Linearity of the detector response verses concentration of analyte is attained over the established calibration ranges tested between 10 to 80 µg/mL and correlation coefficient obtained is greater than 0.999 and a bias of 1.43% for Pemetrexed peak. From results, it is evident that the method covers a satisfactory range towards accuracy and linearity as per current validation practices. Refer Table-4A and 4B.

Table 4A: Results of Accuracy.

Sample name	Added (µg/mL)	Found (µg/mL)	% Recovery Assay*	Average n=3*	% RSD n=3*
Accuracy 50% Spl-1	19.9200	20.0650	100.73		
Accuracy 50% Spl-2	20.0400	20.1240	100.42	100.34	0.42
Accuracy 50% Spl-3	20.1200	20.0971	99.89		
Accuracy 100% Spl-1	40.0800	40.0734	99.98		
Accuracy 100% Spl-2	40.8400	40.9535	100.28	100.29	0.32
Accuracy 100% Spl-3	40.3200	40.5700	100.62		
Accuracy 150% Spl-1	60.1200	60.1484	100.05		
Accuracy 150% Spl-2	59.9200	60.2396	100.53	100.45	0.36
Accuracy 150% Spl-3	60.2000	60.6563	100.76		

* % Recovered and average of n=3 per each level must be within 97% to 103%, % RSD must be ≤ 2.

Table 4B: Results of Linearity.

Linearity Range	Slope	y-intercept	r *	Bias 100%
10 to 80 µg/mL	22237.22	13005.648	0.99966	1.43

* Coefficient of Correlation: Limit: >0.999, Bias at 100% response: within ± 2%

Solution Stability and Mobile Phase Stability

Mobile phase found stable and is visually clear with no precipitation or turbidity and System suitability parameters were passed over a period of 48 hours. Similarity factor and % assay difference of Standard and test solutions stability results were within ±1% till 48 hours, when compared with initial % assay. Refer Table-2B.

Robustness

Under variable chromatographic conditions (flow rate, column temperature), System suitability parameters were passed and found within the acceptance criteria. This proves that the newly developed method is robust and can withstand deliberate changes in chromatographic conditions. Refer Table-5.

Table 5: Results of Robustness.

Parameter	RT*	%RSD (n=5)**	USP Tailing
0.45ml/min flow rate	1.732	0.29	1.25
0.55ml/min flow rate	1.417	0.47	1.24
35ºC Column temperature	1.573	0.36	1.25
45ºC Column temperature	1.496	0.73	1.24

* retention time in minutes, ** For standard peak

CONCLUSIONS

Recent advancement in chromatography techniques and improved understanding on chemistry, degradation behavior and stability of drug substances and drug products made regulatory authorities and ICH guidelines to raise concerns over developing and validating stability-indicating LC methods to evaluate quality through stability testing (ICH Q2 (R1) 2005). The newly developed, rapid simple linear gradient stability indicating RP UPLC method with shortest runtime of 4.0 minutes for determining assay of pemetrexed from parenteral formulation in presence of forced degradation products is specific, precise, accurate, linear and robust. Reproducible and smooth baseline is achieved throughout the work and issues due to Carry over which generally rise during analysis were well resolved. Satisfactory results were obtained from validation of the method as per ICH. This stability-indicating method can be implemented to support analysis of developmental, lab-scale, QC-stability testing and production samples of pemetrexed disodium parenteral formulation.

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