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A Validated and Stability Indicating Ultra High Pressure Liquid Chromatographic Method for Folic Acid in Pharmaceutical Preparation

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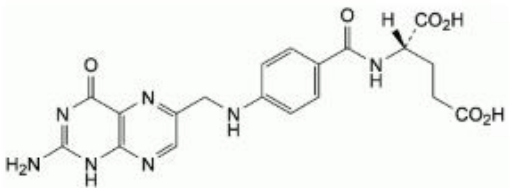
A simple, selective, linear, precise, and accurate ultra-high performance liquid chromatography method was developed, optimised and validated for the quantification of synthetic folic acid (FA) in pharmaceutical dosage form. Isocratic elution at a flow rate of 0.4 mL/min was employed on C8 1.7 µm (2.1 mm x 100 mm) or equivalent column at ambient temperature. The mobile phase consists of Acetonitrile :0.005 M 1–Hexane Sulfonic Acid Salt. PH 2.5 with Phosphoric acid in the ratio of 10:90 v/v. The UV detection wavelength was 210 nm, and5µL sample was injected. The Flow rate was found to be 0.4 ml/min The retention time for folic acid was ± 2.0 min. The percent RSD for accuracy of the method was found to be 0.2%. The correlation coefficient (R^2) for Folic Acid is 1.000. The average percent Recovery is varying from 104.5 – 96.9. The method for the Dissolution of Folic Acid 5 mg Tablets complies with the requirements for Specificity, System suitability, Linearity, Accuracy and Method precision across the range of 25 % to 125 %. The method is therefore acceptable as valid and stability indicating. The method was validated as per the guidelines. The method can be successfully applied for Folic acid in the rapid and reliable determination of folic acid in pharmaceutical dosage form.

Keyword: Folic Acid, UPLC, UV-Dection, % Recovery, Accuracy.

1. Introduction



Folic acid (also known as folate, vitamin, vitamin B_9,[1] vitamin Bc[2] (or folacin) and pteroylmonoglutamic acid[3] are forms of the water-soluble vitamin B_9. Folic acid biological importance is due totetrahydrofolate and other derivatives after its conversion to dihydrofolic acid in the liver.[4]

Folic Acid is a yellowish or orange crystalline powder. Practically insoluble in water and in most organic solvents. For the determination of water-soluble vitamins, various methods such as, volumetric assays[5], spectrometric assays[6], capillary electrophoresis[7], high-performance liquid chromatography (HPLC)[8-11] have been reported. Previous reports[12-15] have demonstrated that the LC–MS–MS provides sufficient sensitivity and selectivity for the determination of vitamins and nicotinic acid. Recently, ultra-performance liquid chromatography (UPLC), which can be operated at ultra-high pressure (for instance 1000 bar), has

been applied[16-17]. According to[18] the individual folates, as their monoglutamyl forms, were separated and measured by HPLC, and the peaks counted for 'H. By the studies[19] the method described an optimized solid phase extraction technique for selective analyte extraction using cartridges containing both lipophilic and cation-exchange properties. The captured analytes are then subjected to UPLC separation, followed by MS/MS analysis using information-dependent acquisitions and SRM. According to the[20] concentrations of the folate forms FA, 5-methyltetrahydrofolate (5-MTHF), and THF were measured before and after 3-week placebo or FA 5 mg, vitamin B6 40 mg, and cyanocobalamin 2 mg per day administrated to 74 older adults (median age, 82 years). Concentrations of unmetabolized FA were positively related to those of 5-MTHF and THF. By the Studies[21] Standard calibration curves for the two analytes were linear over the range of 0.018–14 μg FA/g of fresh bread (r2=0.997) and 9.3–900 ng 5-MTHF/g of fresh bread (r2=0.999). The absolute recoveries were 90% and 76% for FA and 5-MTHF, respectively. Intra-day coefficients of variation were 3% for FA and 18% for 5-MTHF. The limit of detection was 9.0 ng/g for FA and 4.3 ng/g for 5-MTHF. According to[22] HPLC–MS/MS has been established. UPLC was performed under gradient conditions on an Acquity HSS T3 column, followed by tandem mass spectrometry detection. The method was validated based on linearity, sensitivity, precision, accuracy and matrix effects. The LOD and LOQ varied between 0.06 and 0.45_g/100 g and 0.12 and 0.91_g/100 g, respectively. Two linear calibration curves were established, one for the low and the other for the high concentration range. By the studies[23] the quantification limits were between 0.17 nmol/L (5-formylTHF) and 1.79 nmol/L (THF), and the assay was linear up to 100 nmol/L (5-methylTHF) and 10 nmol/L (5-formylTHF, 5,10- ethenylTHF, THF, and folic acid). Mean recoveries were between 82.3% for THF and 110.8% for 5,10-methenylTHF. Quantitative fatty acid[24] composition of microorganisms at various growth space points is required for understanding membrane associated processes of cells, but the majority of the relevant publications still restrict to the relative compositions. The various assays have been validated[25] for intra- and inter-run precision, accuracy, linearity and are robust. The present assays are robust and allow for high-throughput analysis. The starting conditions for the development were calculated starting from the HPLC conditions of a validated method [26]. Therefore, a novel multi-vitamin analysis method using ultra-performance liquid chromatography (UPLC) tandem mass spectrometry was developed in this paper

2. Materials and Methods

2.1 Chemicals, Instrument and reagents:

The sample of Folic Acid 5mg tablets was obtained from Ranbaxy, Mumbai. 1–Hexane Sulfonic Acid Salt, Phosphoric acid and Acetonitrile used were of HPLC grade and purchased from Merck Specialties Private Limited, Mumbai, India and orthophosphoric acid is AR Grade purchased from Local market. Variable wavelength programmable UV-Visible detector and Rheodyne injector was employed for investigation. The chromatographic analysis was performed on a C8 1.7 μm (2.1 mm x 100 mm). Degassing of the mobile phase was done using a Loba ultrasonic bath sonicator. A Denwar Analytical balance was used for weighing the materials.

2.2 Preparation of Standard Solution:

Accurately weigh 55 mg of Folic Acid into a 100 ml volumetric flask. Add about 60 ml of solvent sonicate for 5 minutes to dissolve. Make up to volume with solvent and mix well. Dilute 10 ml of this solution to 50 ml volumetric flask and dilute to volume with mobile phase. Further dilute 5 ml of this solution to a 50 ml volumetric flask and make up to the mark with mobile phase.

2.3 Preparation of Sample solution:

Using 500 ml of filtered and degassed dissolution medium maintained at 37 °C. Place one tablet into the dissolution vessels and immediately operate the apparatus at 50 rpm. Withdraw 20 ml

samples after 45 minutes. Filter the samples through a 0.2 µl filter.

2.4 Chromatographic Conditions:

Experiment was run with the mobile phase consists of Acetonitrile: 0.005 M 1–Hexane Sulfonic Acid Salt. P^H 2.5 with Phosphoric acid in the ratio of 10:90 v/v. Methanol: Acetonitrile: 1% OPA 80:18:2 v/v/v C8 1.7 µm (2.1 mm x 100 mm) or equivalent. 20 µl of sample is injected with a flow rate of 0.4ml/min and effluents were identified at 210nm. The sample retention time is ± 2.0 Min.

2.5 Method of Analysis – Assay:

Dissolution of Folic Acid NLT 75 % (Q) in 45 minutes was determine the content of
Folic Acid, by following the procedure for External Standard UPLC under the dissolution conditions. The total No. of Tablets used is 6. Dissolution medium, volume is Water and 500 ml respectively. The Stirrer speed 50 rpm and Withdrawal volume is20 ml. And we are protected all the solutions from the light.

2.6 Method Development:

For developing the method, a systematic study of the effect of various factors was undertaken by varying one parameter at a time and keeping all other conditions constant

2.7 Wavelength Detection:

The spectrum Folic acid in methanol was recorded separately on UV spectrophotometer. The peak of maximum absorbance wavelength was observed. The spectra of Folic acid were showed maximum absorbance at 210nm.

2.8 Validation of the proposed method:

The analytical performance of the method of analysis was checked for Specificity, System suitability, Accuracy and Method Precision.

2.9 Specificity:

Specificity of an analytical procedure is its ability to assess unequivocally the analyte in the presence of components that may be expected to be present. The requirements for this method are the solvent and placebo solutions must contain no components, which co-elute with the Folic Acid. The peak purity results from the photo diode-array analysis must show that the Folic Acid peak is pure – i.e. the purity angle (PA) must be less than the threshold angle (TH). The solutions listed below were injected using the conditions specified in the method of analysis. Peak purity Graphs obtained were shown in the fig.7 to 11. And results were shown in the Table: 3. Chromatographic results were taken from assay method validation. (MV/AUP/18). Chromatogram peaks were shown in the fig: 2 to 6.

Table: 1

1.	Solvent – Mobile phase
2.	Drug active – Folic Acid at working concentration
3.	Product at working concentration
4.	Active, product stressed under UV light for 72 hours
5.	Placebo at working concentration

Table: 2

1.	Solvent – No significant peak detected	Chromatogram 1
2	Drug active, product – Peak due to Folic Acid eluted at 1.8 minutes.	Chromatogram 2 & 3 respectively
3.	Active, product – UV stress: Peak due to Folic Acid eluted at 1.8 minutes.	Chromatogram 4 & 5 respectively
4.	Placebo – No significant peak detected.	Chromatogram 6

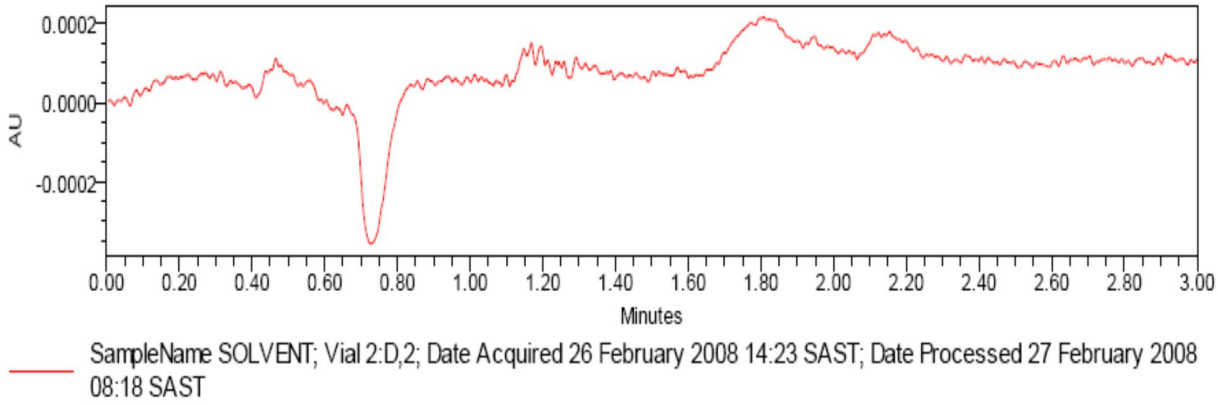


Fig: 2: Chromatogram 1

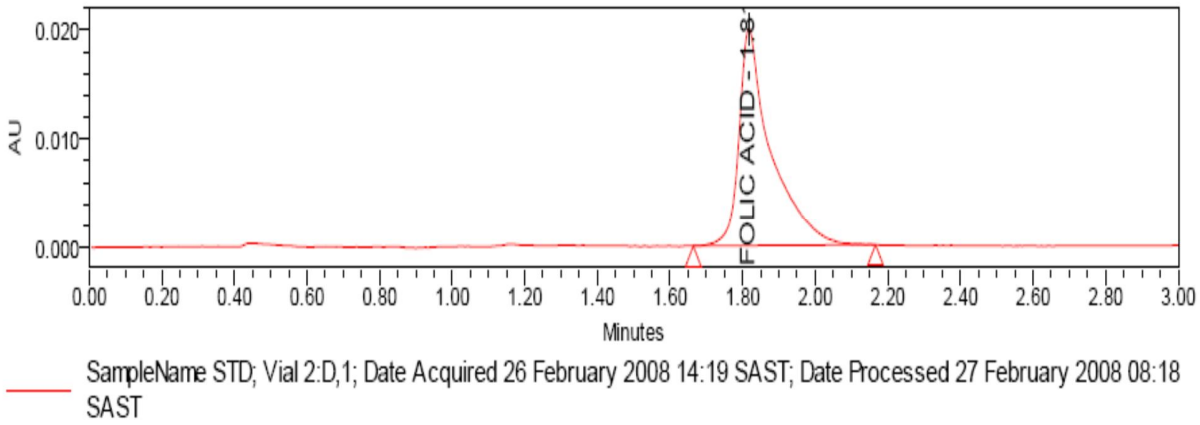


Fig: 3: Chromatogram 2

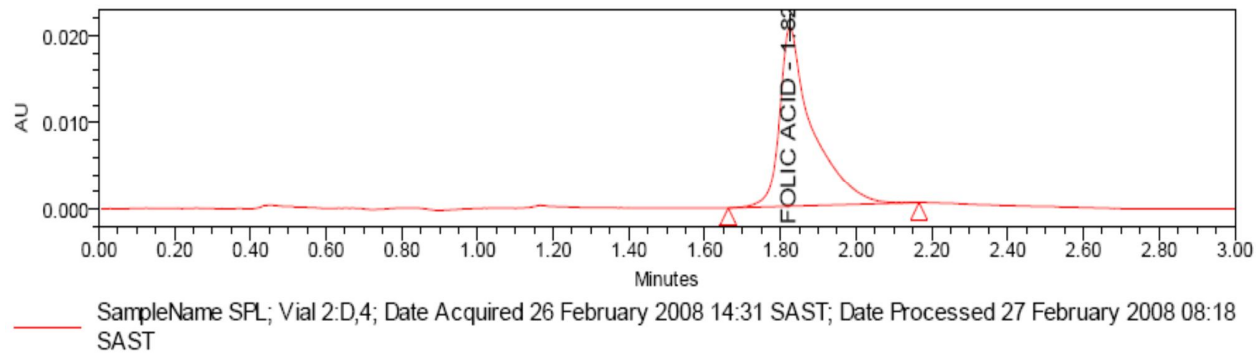


Fig: 4: Chromatogram 3

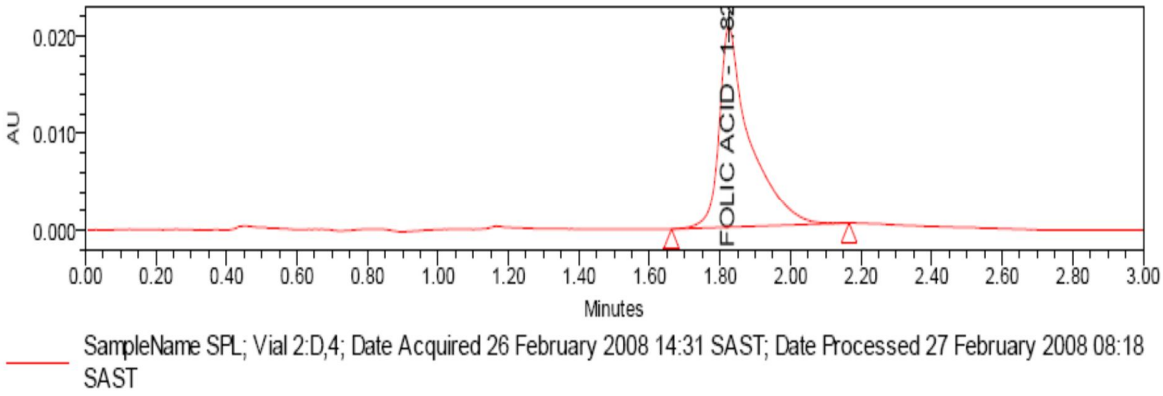


Fig: 5: Chromatogram 4

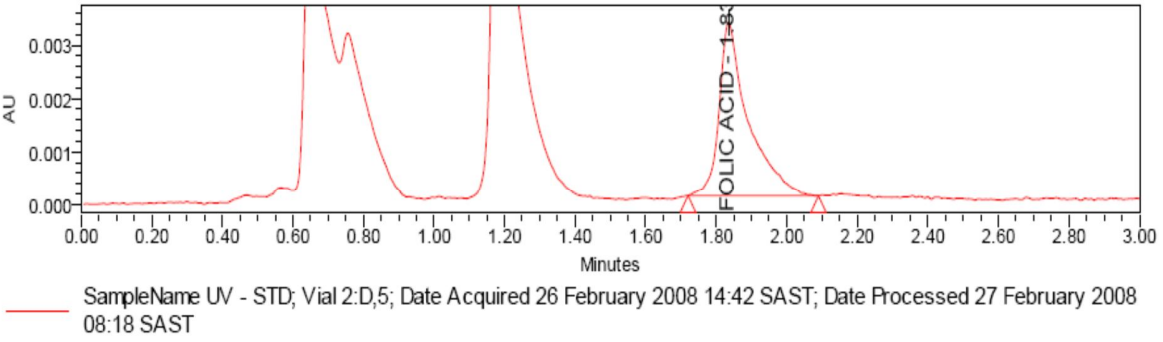


Fig: 6: Chromatogram 5

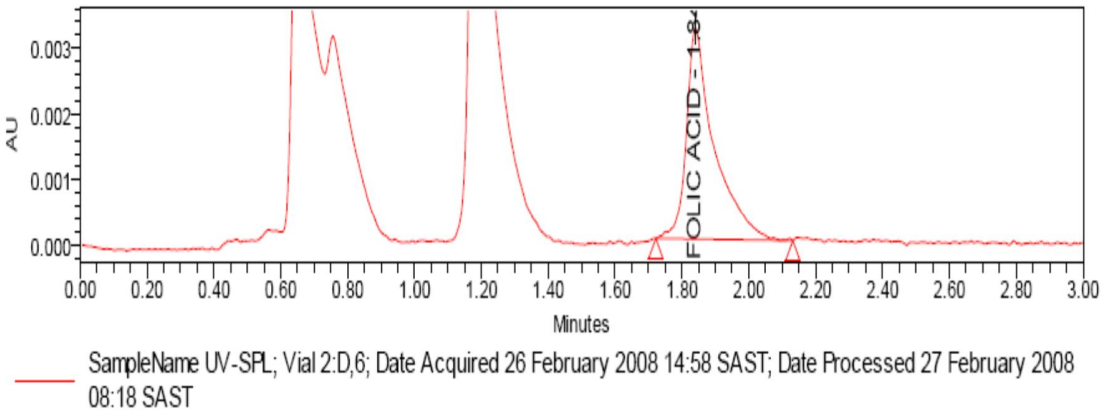


Fig: 7: Chromatogram 6

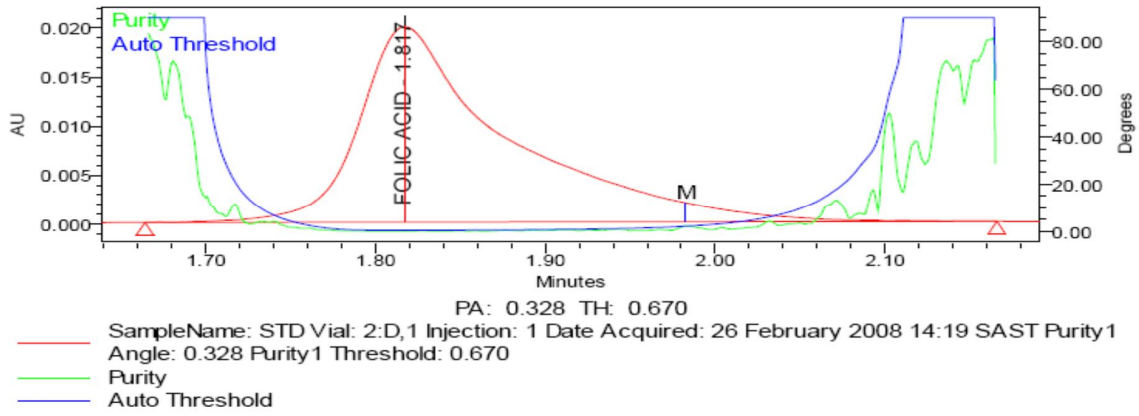


Fig 8: Peak Purity 1

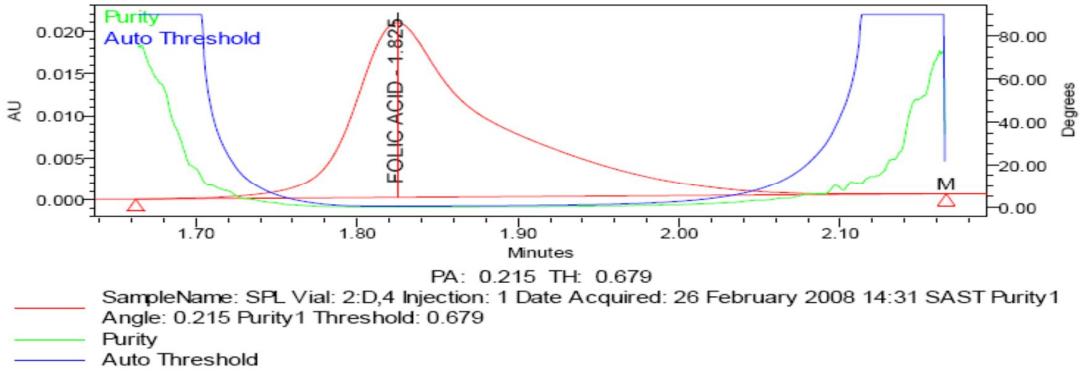


Fig 9: Peak Purity 2

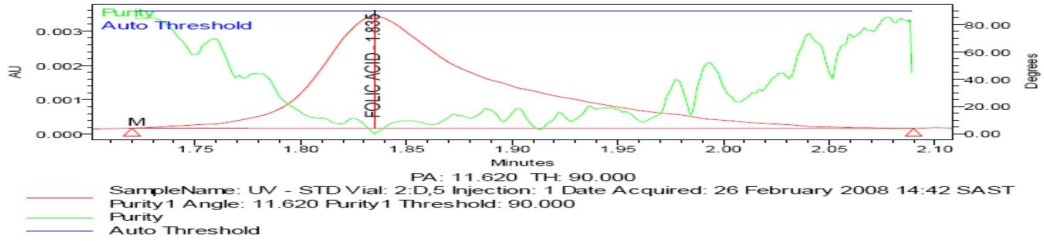


Fig 10: Peak Purity 3

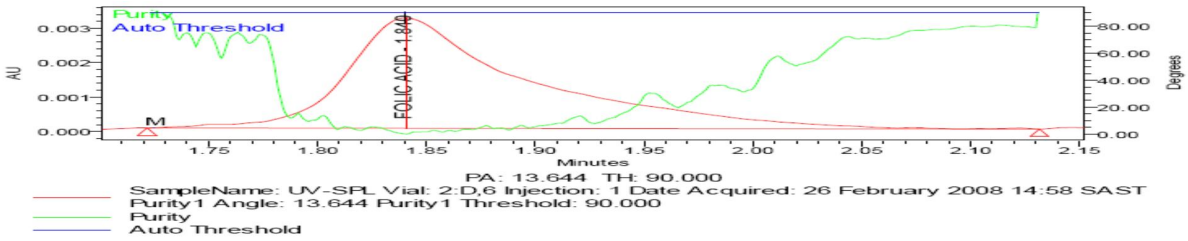


Fig 11: Peak Purity 4

Table 3: Peak Purity Results

Folic Acid			
	Purity angle < Threshold		
1.	Drug active	0.328 < 0.670	Peak Purity 1
2.	Drug product	0.215 < 0.679	Peak Purity 2
UV - STRESSED			
	Purity angle < Threshold		
3.	Drug active	11.620 < 90.000	Peak Purity 3
4.	Drug product	13.644 < 90.000	Peak Purity 4

Folic Acid is not stable under UV light exposure as it shows degradation. No components are seen to co-elute with peaks, and the peak purity results indicate that Folic Acid peak can therefore be considered spectrally pure.

3. System Suitability:

System suitability is a measure of the performance and chromatographic quality of the total analytical system – i.e. instrument and procedure. The requirements for the measurement of this method are the % RSD of the peak responses due to Folic Acid for the six replicate injections must be less than or equal to 2.0 %. Six replicate injections of working standard solution were injected according to the method of analysis. The percentage relative standard deviation (% RSD) for the peak responses was determined. The analytical system complies with the requirements specified by the system suitability. The results obtained were tabulated in the Table: 4.

Table: 4

Sample	Folic Acid Area
1	442233
2	444449
3	443954
4	443311
5	444269
6	443412
Mean	443605
% RSD	0.2

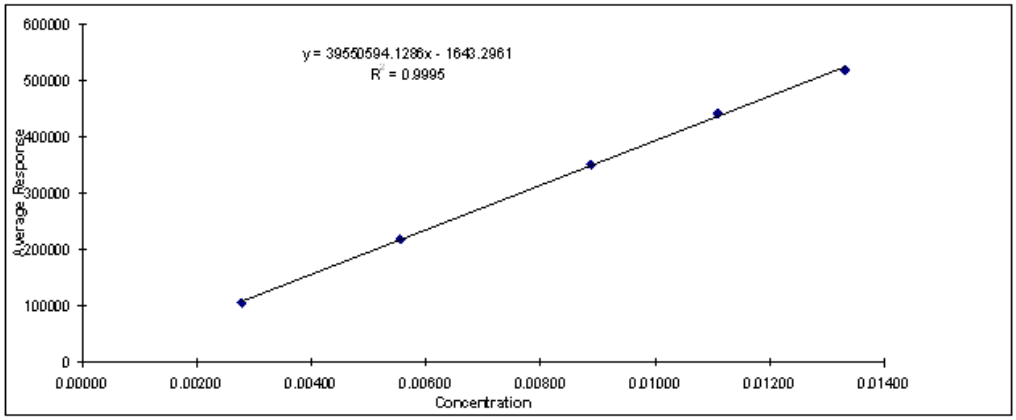
4. Linearity:

The linearity of an assay method is its ability to elicit test results, which are directly proportional to the concentrations of drug actives in samples in a given range. Proof of linearity justifies the use of single-point calibrations. The requirements for the linearity are the correlation coefficient of the regression line for Folic Acid should be greater than or equal to 0.999. The Y-intercept of the line should not be significantly different from zero, i.e. the assessment value (z) falls within the specified limits only when $+2 > z > -2$. Five solutions containing 25, 50, 75, 100, and 125 % of Folic Acid, relative to the working concentrations, were prepared and injected according to the method of analysis. A linear regression curve was constructed, and the correlation coefficients (R^2) and assessment values calculated. The correlation coefficient (R^2) for Folic Acid is 1.000.The plot is a straight line, and the assessment value (z) is 0 for Folic Acid. the Linearity results were shown in the Fig: 12.

Fig 12: Linearity Results

Sample Number	Concentration	Response 1	Response 2	Average Response
25%	0.00278	106322	105543	105933
50%	0.00555	217943	218756	218350
75%	0.00888	351585	351388	351487
100%	0.01110	443081	441247	442464
125%	0.01332	519739	520550	520145

Coefficient of determination (R) = 0.995
Coefficient of Correlation (R2) = 0.995
Acceptance Criterion: The assessment Value (Z) falls within the specified limits only when +2 > z> -2.
Calculated assessment of (z):
A = 1643.2961
100% response = 351487
Z = 0



Calibration Curve: Y = Bx+A, R= Coeff.of Determination

5. Accuracy:

The accuracy of an analytical method expresses the closeness of test results obtained by that method to the true value. The percentage recovery of the active compounds, for each solution prepared, must be within 95.0 – 105.0 % of the actual amount. Sample solutions were spiked with known concentrations of Folic Acid to result in concentrations representing respectively 25, 50, 75,100, and 125 % of Folic Acid relative to the working concentrations. The above samples were injected in duplicate according to the method of analysis. From the accuracy results above, the percentage recovery values for Folic Acid satisfy the acceptance criteria for accuracy across the range of 25 % - 125 %. The results were tabulated in the Table: 5.

Table: 5

Sample	Theoretical	Actual	% Recovery	Average % Recovery
25 %	24.17	23.35	96.6	96.9
25 %	24.17	23.50	97.2	
50 %	48.33	50.65	104.8	104.5
50 %	48.33	50.36	104.2	
75 %	72.50	74.10	102.2	102.1
75 %	72.50	73.87	101.9	

100 %	96.66	96.88	100.2	100.2
100 %	96.66	96.72	100.1	
125 %	120.83	120.66	99.9	99.9
125 %	120.83	120.63	99.8	

6. Method Precision:

The precision of an analytical procedure expresses the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of a homogenous sample.

5.1 Repeatability:

This parameter determines the repeatability of assay results under the same operating conditions over a short period of time. The requirements for the Repeatability tests are the % RSD due to Folic Acid concentration for the six samples must be less than or equal to 5.0 %. Six separate sample preparations of batch 247698 were analysed according to the method of analysis. The % RSD due to Folic Acid concentration for the assay meets the requirements for repeatability at 3.2 % respectively. The results were tabulated in

Table 6:

Sample number	Results (%)
1	103
2	99
3	96
4	96
5	97
6	94
Mean	98
% RSD	3.2

7. Intermediate Precision :

Intermediate Precision of an analytical procedure expresses intra-laboratory variations of the repeatability test performed by a different analyst, on a different day, and using different reagents, mobile phases and solvents. The % RSD due to Folic Acid concentration for the six samples must be less than or equal to 5.0 %. The mean results obtained in the repeatability, and the intermediate precision must not differ by more than 5.0 %. Six separate sample preparations of batch 247698 were assayed according to the method of analysis. The % RSD for intermediate precision is 4.7 %.The intermediate precision and repeatability complies as they differ by 1.4 %. The results were tabulated in Table: 7.

Sample	Results (%)
1	100
2	94
3	100
4	97
5	100
6	108
Mean	100
% RSD	4.7

8. Range:

Range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. Based on the accuracy results, the range for the dissolution of Folic Acid 5 mg Tablets is 25 – 125 % of Folic Acid, which represents 25 % to 125 % of the working concentration.

9. Conclusion

The method for the Dissolution of Folic Acid 5 mg Tablets complies with the requirements for Specificity, System suitability, Linearity, Accuracy and Method precision across the range of 25 % to 125 %. The method is therefore acceptable as valid and stability indicating.

10. References

1. Ural, Serdar H. (2008-11). "Folic Acid and Pregnancy.". Kid's Health.
2. "Definition of vitamin Bc". Medical-dictionary.thefreedictionary.com. Retrieved 2012-09-09.
3. Mayo Clinic Web Editorsolate.http://www.mayoclinic.com/health/folate/NS_patient-folate/DSECTION=synonyms (accessed Nov 15, 2010), additional text.
4. Bailey SW, Ayling JE (September 2009). "The extremely slow and variable activity of dihydrofolate reductase in human liver and its implications for high folic acid intake".Proceedings of the National Academy of Sciences of the United States of America 106 (36):154249.doi:10.1073/pnas.0902072106. PMC 2730961.PMID 19706381.
5. S. Kwon, P.C. Lee, E.G. Lee, Y.K. Chang, and N. Chang. Production of lactic acid by Lactobacillus rhamnosus with vitamin-supplemented soybean hydrolysate. Enzyme Microb. Techol. 2009, 26: 209.
6. Z.O. Tesfaldet, J.F. Van Staden, and R.I. Stefan. Sequential injection spectrophotometric determination of iron as Fe(II) in multi-vitamin preparations using 1,10-phenanthroline as complexing agent. Talanta 2004, 64: 1189.
7. S. Mou, X. Ding, and Y. Liu. Separation methods for taurine analysis in biological samples. J. Chromatogr. B, 2002, 781: 251.
8. P. Moreno and V. Salvado. Determination of eight water- and fat-soluble vitamins in Multi-vitamin pharmaceutical formulations by high-performance liquid chromatography. J. Chromatogr. A, 2000, 870: 207.
9. P.F. Chatzimichalakis, V.F. Samanidou, and I.N. Papadoyannis. Development of a validated liquid chromatography method for the simultaneous determination of eight fat-soluble vitamins in biological fluids after solid-phase extraction. J. Chromatogr. B: Anal. Technol. Biomed. Life Sci., 2004, 805: 289.
10. B. Klejdus, J. Petrlova, D. Potesil, V. Adam, R. Mikelova, J. Vacek, R. Kizek, and V. Kuban. Simultaneous determination of water-and fat-soluble vitamins in pharmaceutical preparations by high-performance liquid chromatography coupled with diode array detection.Anal. Chim. Acta, 2004, 520: 57.
11. A. Zafra-Goamez, A. Garballo, J. C. Morales, and L. Garciaa-Ayuso. Simultaneous determination of eight water-soluble vitamins in supplemented foods by liquid chromatography. J. Agric. Food Chem. 2006, 54: 4531.
12. Z. Chen, B. Chen, and S. Yao. High-performance liquid chromatography– electrospray ionization mass spectrometry for simultaneous determination of taurine and 10 water-soluble vitamins in multivitamin tablets. Anal. Chim. Acta, 2006, 569: 169.
13. X. Luo, B. Chen, L. Ding, F. Tang, and S. Yao. HPLC–ESI-MS analysis of vitamin B12 in food products and in multivitamins-multimineral tablets. Anal. Chim. Acta 2006, 562: 185.
14. B. C. Nelson, K. E. Sharpless, L.C. Sander. Quantitative determination of folic acid in multivitamin/multielement tablets using liquid chromatography–tandem mass spectrometry. J. Chromatogr. A, 2006, 1135: 203.
15. R. Mittermayr, A. Kalman, M. Trisconi, O. Heudi. Determination of vitamin B5 in a range of fortified food products by reversed-phase liquid chromatography–mass spectrometry with electrospray ionization. J. Chromatogr. A, 2004, 1032: 1.
16. H. Kawanishi, T. Toyooka, K. Ito, M. Maeda, T. Hamada, T. Fukushima, M. Kato, and S. Inagaki. Rapid determination of histamine and its metabolites in mice hair by ultra-performance liquid chromatography with time-of-flight mass spectrometry. J. Chromatogr. A, 2006, 1132: 148.
17. E. Barcelo-Barrachina, E. Moyano, M.T. Galceran, J.L. Lliberia, B. Bago, and M.A. Cortes. Ultra-performance liquid chromatography–tandem mass spectrometry for the analysis of

heterocyclic amines in food. J. Chromatogr. A, 2006, 1125: 195.
18. Karen C. Scott and Jesse F. Gregory III (The fate of [3H]folic acid in folate-adequate rats - J. Nutr. Biochem., 1996,7:261-269.
19. Azeret Zuniga, Liang Li. (Ultra-high performance liquid chromatography tandem mass spectrometry for comprehensive analysis of urinary acylcarnitines-Analytica Chimica Acta, 2011, 689; 77–84.
20. Concentrations of unmetabolized folic acid and primary folate forms in plasma after folic acid treatment in older adults - Metabolism Clinical and Experimental, 2011, 60 673–680.
21. Folate analysis in foods by UPLC-MS/MS: development and validation of a novel, high throughput quantitative assay; folate levels determined in Australian fortified breads- Anal Bioanal Chem, 2011; 401:1035–1042.
22. Ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) for the sensitive determination of folates in rice - Journal of Chromatography B, 2010, 878; 509–513.
23. Quantification of key folate forms in serum using stable-isotope dilution ultra performance liquid chromatography–tandem mass spectrometry, Journal of Chromatography B, 2010, 878; 68–75
24. UPLC/MS based method for quantitative determination of fatty acid composition in Gram-negative and Gram-positive bacteria , Journal of Microbiological Methods, 2010 82; 288–295.
25. Comprehensive quantitativemeasurement of folate polyglutamates in human erythrocytes by ion pairing ultra-performance liquid chromatography/tandem mass spectrometry- Rapid Commun. Mass Spectrom. 2012, 26, 1617–1630.
26. A validated ultra high pressure liquid chromatographic method for qualification and quantification of folic acid in pharmaceutical preparations - Journal of Pharmaceutical and Biomedical Analysis, 2011, 54; 995–1000.