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Validation of High Performance Liquid Chromatographic Method for Folic Acid Assay

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Abstract: *A high performance liquid chromatography (HPLC) was developed and validated as selective, linear, precise and accurate method for estimation of folic acid (FA). The reversed C_{18} column (Kromasil 100-5 phenyl® 300 X 4.6 mm, 5µm) was employed at 25°C. The mobile phase was 0.1% v/v trifluoroacetic acid (TFA) and acetonitrile at ratio 80:20 and the flow rate 1.5ml /min. The retention time for folic acid was ± 2.0min. The method showed excellent recovery (99.04%) and precision for determining the folic acid at low concentrations reaching 0.1µg/mL Therefore, the method was accepted as valid stable assay for determination the folic acid in different pharmaceutical dosage forms.*

Keywords: *Accuracy, Folic acid, HPLC, Linearity, Validation.*

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1. Introduction

Folic acid (FA) is a yellowish or orange crystalline powder with a limited solubility in most organic solvents and insoluble in water [1]. The chemical name is *N*-[4-2-amino-3, 4-dihydro-4-oxo-6-pteridinyl) methyl amino benzyl- L-glutamic acid [2] and the molecular weight is 441Da (Fig. 1). It posses numerous advantages including; stability over a broad range of temperature and pH values, inexpensive, nontoxic and nonimmuogenic [3]. Folic acid has extensive attention as targeting ligands due to its low molecular weight, ease of conjugation with nanoparticles and high specificity to folate receptors ($Kd=10^{-10}$ M)[4].

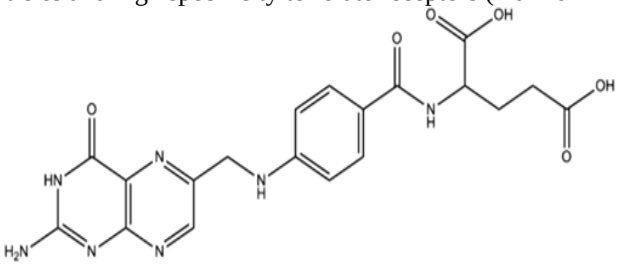

Figure 1: Molecular structure of folic acid

Prior for using FA in nanoparticles formulation, it should be successfully able to couple with the used polymer at high coupling ratio. For estimating the coupling ratio of FA and the polymers, FA assay should be carried out. Different analytical methods was reported for determining FA including; volumetric assays, spectrometric assays [5] capillary electrophoresis [6], conventional chromatographic procedure such as thin-layer column chromatography (TLC) and high performance liquid chromatography (HPLC) [7]. Applying HPLC method is gained more acceptances because of its abundant advantages; more rapid, accurate, sensitive and selective assay yielding a better resolution [8]. Moreover, it is an automatic method applied to several categories of substances: carbohydrates, lipids, vitamins, additives, synthetic colorings, natural pigments, contaminants, and amino acids. It has different ways for separation depending on the nature of the stationary phase including: adsorption chromatography, partition chromatography, ion-exchange chromatography, and size exclusion chromatography [9]. This study was aimed to develop and validate a specific HPLC method for

assaying FA after its conjugation with chitosan. The C-18 column and reverse phase conditions were evaluated in combination with UV detection for efficient and accurate quantification of FA.

2. MATERIALS AND METHODS

2.1. Materials

Folic acid (FA) was procured from BDH Chemicals, England. Anhydrous dimethyl sulfoxide (DMSO) was purchased from Fluka Chemicals, Switzerland. Acetone, acetonitrile, trifluoroacetic acid (TFA) and methanol (HPLC grade) were purchased from Riedel-de Haen Gmbh, Germany.

2.2. Chromatographic conditions

A HPLC system consisting of Agilent 1100 controlled by chem. station Data System and equipped with G 1311A quaternary pump and UV detector (VWD-G1314 A) was employed for investigation. A reverse phase C_{18} column (Kromasil 100-5 phenyl®, 300X4.6 mm, 5 µm) was used at 25°C. The experiment was run with mobile phase consisted of 0.1% v/v TFA and acetonitrile at ratio (80:20 v/v). It is injected with flow rate 1.5ml/min and the elute was monitored at wavelength 290 nm.

2.3. Analytical validation

The analytical performance of the method of analysis was checked for selectivity, linearity, range, recovery, accuracy, precision, limit of detection and limit of quantitation [10].

2.3.1. Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample.

2.3.2. Linearity and range

Linearity was determined by calculating the regression line using a mathematical treatment of the peak area versus folic acid concentration in 2% v/v acetic acid. The range of the method is the interval between the upper and lower levels of an analyte concentration that have been determined with acceptable precision, accuracy and linearity. It was determined on a linear response curve and was expressed in the same units as the test results.

2.2.3. % Recovery

The % recovery was assessed by dissolving known amounts of folic acid in 2%v/v acetic acid to give concentrations of 0.1, 0.2, 0.5, 1, 1.5 and 2µg/mL. Recovery percentage was calculated by comparing the obtained concentration with the theoretical concentrations.

2.2.4. Accuracy and precision

Assay performance was evaluated according to intra- and inter-day accuracy and precision. Replicate analysis of control specimens at the concentration range of 0.1-2µg/mL on a single day was assessed within-day assay (intra-day).

Inter-day reproducibility was determined by comparing the data obtained from samples analysis for three successive days. Accuracy is a measure of the closeness of test results to the theoretical value. The % accuracy was determined by using the following equation (1):

$$\text{Accuracy} = \left(\frac{\text{Theoretical Conc.} - \text{Measured Conc.}}{\text{Theoretical Conc.}}\right) * 100 \qquad (1)$$

Precision is a measure of the reproducibility of the whole analytical method (including sampling, sample preparation and analysis) under normal operating circumstances. Precision was determined by repeating the method of assay 6 times and was then expressed as coefficient of variation (C.V. %).

2.2.5. Limit of detection (LOD)

The LOD is the lowest concentration of a sample that can be detected but not necessarily quantitated. It was quoted as the concentration yielding a signal-to-noise at ratio 3:1 (height of the peak corresponding to the component/absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution) [11].

2.2.6. Limit of quantitation (LOQ)

The LOQ is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy. It was quoted as the concentration yielding a signal-to-noise at ratio 10:1[12].

III. RESULTS AND DISCUSSION

3.1. HPLC assay of folic acid

The HLPC assay of folic acid developed for estimating the concentration of folic acid conjugated with chitosan polymer. For this purpose, various mobile phases with different compositions were tried namely

methanol: water, methanol: phosphate buffer pH 3.5, 5.5, 6.8, 7, 7.4, methanol: 0.1%, 0.5 v/v TFA, acetonitrile: water, acetonitrile: phosphate buffer pH 3.5, 5.5, 6.8, 7 or 7.4, acetonitrile: 0.1%, 0.5 v/v TFA. At wavelength of 290 nm, the best peak separation was obtained using 0.1% v/v TFA and acetonitrile (80:20 v/v).

3.2. Validation of the HPLC method

3.2.1. Selectivity

The FA chromatogram at concentration (5µg/mL) in 2% acetic acid was illustrated in Fig. 2. The FA was well separated with a mean retention time of 2.79min. The FA peak was sharp and symmetric with good base line with no tailing or peak splitting. The obtained chromatogram allows an accurate peak area calculation.

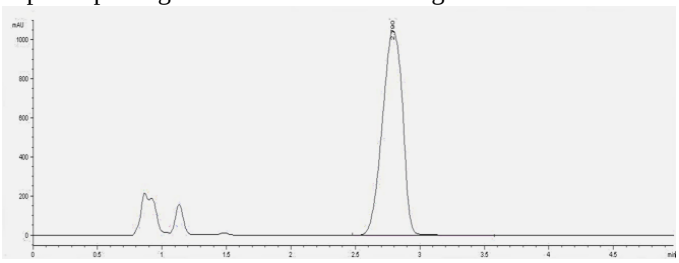

Figure 2: Representative chromatogram of folic acid (5µg/mL) in 2% v/v acetic acid.

3.2.2. Linearity and range

Standard plots obtained for FA in acetic acid (2% v/v) were linear in the range of 0.1-5µg/mL Fig. 3. Linear regression analysis of the standard calibration plots is y=5371.8x + 301.13 where y and x are peak area and FA concentration, respectively. The coefficient of determination R^2 value was found to be 0.9994.

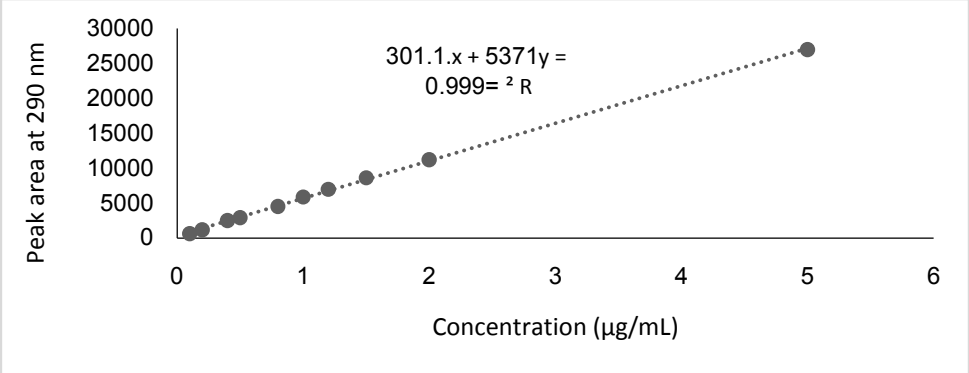

Figure 3: Calibration curve of folic acid in 2% v/v acetic acid.

3.2.3. Recovery

The %recovery for FA obtained using a mixture of 0.1% v/v TFA and acetonitrile (80:20 v/v) is shown in Table 1. The average of % recovery of FA in 2% v/v acetic acid was nearly 99%.

Table 1: Recovery of folic acid from 2% v/v acetic acid

Spiked conc. (µg/mL)	**Mean conc.* (µg/mL ±SD)**	**Recovery%**
0.1	0.098	98.82
0.2	0.19	99.89
0.4	0.39	99.85
0.5	0.49	98.48
0.8	0.79	99.21
1	0.99	99.11
1.2	1.18	99.09
1.5	1.49	99.63
2	1.94	97.06
5	4.96	99.22

* mean of six measurements ± standard deviation

3.2.4. Accuracy and precision

The closeness between the test results and their true values expresses the accuracy of an analytical method [1]. Data concerning intra- and inter-day accuracy and precision were determined and are listed in Tables 2 & 3. The results showed intra-day accuracy in range 0.105 - 2.93, while the respective values of precision expressed as % C.V. was 0.87 - 3.36. Inter-day accuracy values were in the range of 0.03 -1.96. The calculated % C.V. values were found to be in the range 1.11 to 9.98 denoting good precision.

Table 2: Intra-day accuracy and precision for folic acid in 2% v/v acetic acid

Spiked conc. (µg/mL)	**Mean conc.* (µg/mL ±SD)**	**Accuracy**	**C.V. %****
0.1	0.098	1.17	3.36
0.2	0.19	0.10	2.23
0.4	0.39	0.14	1.97
0.5	0.49	1.51	1.93
0.8	0.79	0.78	2.94
1	0.99	0.88	1.33
1.2	1.18	0.90	2.04
1.5	1.49	0.36	0.87
2	1.94	2.93	1.55
5	4.96	0.77	1.76

Table 3: Inter-day accuracy and precision for folic acid in 2% v/v acetic acid

Spiked conc. (µg/mL)	**Mean conc.* (µg/mL ±SD)**	**Accuracy**	**C.V. %****
0.1	0.098±0.004	1.92	4.43
0.2	0.19±0.01	0.42	6.79
0.4	0.39±0.03	1.08	9.98
0.5	0.49±0.01	0.36	2.23
0.8	0.79±0.02	0.56	2.50
1	0.98±0.02	1.96	2.19
1.2	1.19±0.01	0.17	1.11
1.5	1.48±0.02	0.91	1.83
2	1.99±0.07	0.03	3.86
5	4.95±0.16	0.98	3.42

* mean of six measurements ± standard deviation
** C.V%= coefficient of variation %

3.2.5. Limit of detection and limit of quantitation

The limit of detection (LOD) corresponding to the lowest concentration that produced a peak height approximately triple the baseline noise was 0.05 µg/ mL. The LOQ of folic acid was 0.1µg/mL presents the lowest tested concentration at which both accuracy and precision were within the proposed criteria.

IV. CONCLUSION

The HPLC method for assaying of FA complies with the requirements for selectivity, linearity, recovery, accuracy, precision, limit of detection and limit of quantitation. Therefore this method is acceptable as valid and stable assay.

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