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Bioanalytical method development and validation of folic acid from rat plasma using reverse phase high performance liquid chromatography

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ABSTRACT: A simple, rapid and sensitive Reverse phase High performance liquid chromatography (RP-HPLC) bioanalytical method was developed for the determination of folic acid in rat plasma using methylparaben as an internal standard. The developed method involves simple sample preparations and had total run time of 20 min using RP-HPLC. Chromatographic separation was performed on Inertsil ODS C18 column (4.6×100 mm, 5µm) using isocratic elusion system of potassium phosphate buffer (pH 7): Methanol (75:25 v/v) as mobile phase. Mobile phase flow rate was kept at 1ml/min with column temperature of 40°Cat pressure 88-90 bars. The maximum wavelength used for the detection of folic acid was 283nm. The retention time of folic acid and methylparaben was found to be 6.4 & 13.2 respectively. The method was tested for linearity range of 2 to 12 µg/ml. Developed method was validated for system suitability, selectivity, linearity & range, accuracy, precision, robustness, LOD & LOQ, and stability according to the international regulatory guidelines. Minimum sample preparation and short run time make the method valuable for performing the analysis. Developed method was successfully applied in the determination of folic acid levels *In-vivo* in rat plasma after oral administration.

KEYWORDS: RP-HPLC, folic acid, methylparaben, validation, plasma

1. INTRODUCTION

Analysis of any product is important to understand the quality as well as quantity of active material present in it, but in case of drug substances that may be synthetic or natural origin it is vital, because it directly involves relation to the human life. Hence, there is emerging pharmaceutical branch called 'pharmaceutical analysis' was born to deal with separation, quantification & additive identification of synthetic as well as natural substances with one or more compounds [1]. It is primary task to estimate analysis of pharmaceutical product by developing the analytical method but, with developing the analytical method it is important to validate that particular method, this method can be optimized & validate by taking the trial runs on the specific chromatographic instruments [2].

Method validation is part of process used to confirm the analytical method employed for specific test is suitable for its intended use. The output from the analytical validation can be used to judge the reliability, quality & consistency of the obtained results. To perform the validation of analytical method precisely, some authorities like United States pharmacopoeia (USP), International Council for Harmonization (ICH) and Food and drug administration (FDA) also provide framework for validation of pharmaceutical methods [3,4]. The overall validation process states about the measures of correct drug, in the accurate amount and in the appropriate range for the samples. It helps to analyst to understand the behavior of developed method [5,6]. When these pharmaceutical products or actives delivered in to body it gets contact with blood and other matrix like plasma, urine, serum system so, to detect the amount of any drug or product in to the blood and other matrix, it also required suitable chromatographic method called 'Bio-analytical method.

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Bioanalytical method validation plays an important role of quantitative finding of analytes in biological as well as physiological matrices. Sometimes this method is also applied to study the clinical pharmacology. It could help to evaluate & interpret the pharmacokinetics, bioequivalence and toxicological results. It is the best way by which the drugs or its metabolites efficacy & side effects value can be known performing the sensitive and accurate method development and validation [7,8]. These different studies support the regulatory filings which mean the submission of drug dossier to specific country where we have to launch our product. Without permission of regulatory bodies we are unable to submit the dossier or launch our Pharmaceutical product. More quality work relates to more quality bioanalytical data [9]. Folic Acid (FA) is categorized in water soluble B complex vitamin therefore it is also refer as vitamin B9 or folate. Folic acid is the synthetic, oxidised and simple form of folate and there are more than 100 compounds that can be defined as folate. Folates can be store in the body as they are present in the organ like kidney, liver, tongue etc. whereas, in fruits and vegetables like citrus fruits, leafy green vegetables, bean wheat germ folate is present. Depending on sex, age and country to country variation, the daily intake of folates changes from 100 to 600 µg per day. According to the United State Public Health Service (USPHS), woman of childbearing age should take daily dose 400 µg of folic acid which could prevent the disease like spina bifida and neural tube defects. For breastfeeding woman European government recommend the daily intake of 500 µg folic acid. Folic acid also plays main role in human nutrition as they necessary for the growth, reproduction and normal body function.

It has group of structurally related compounds with same basic skeleton but, differs in their oxidation state & number of glutamate moieties present. Generally, two reduced form of folic acid which are 7,8-dihydrofolate (DHF) and 5,6,7,8-tetrahydrofolate (THF) actually goes in to the blood circulation i.e. to exert biological activity, FA should be reduced to these THF forms. The conversion of folic acid to THF mainly takes in the liver and intestinal mucosa. Folic acid is available in two forms namely natural and synthetic form. The synthetic folic acid is used in the oral supplements or in the fortified food. For the biochemical reactions of DNA and amino acid metabolism the enzyme present called 'folate enzyme' work as single carbon donor and acceptor. [10,11]. The IUPAC name of FA is (2S)-[4-[(2-amino-4- hydroroxypteridin-6-yl) methyl amino] benzamido] glutamic acid with Molecular formula, C19H19N7O6 and Molecular weight is 441.4 g/mol. It is yellow to yellowish orange in color. Although, it is water soluble vitamin but, it is practically insoluble in cold water, slightly soluble in hot water, soluble in diluted acids and alkalies. The monograph of FA is officially given in IP 2007, BP 2009 and Martindale [12,13]. The chemical structure of Folic acid is given (Figure 1).

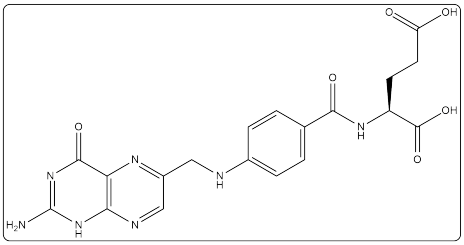

Figure 1. Chemical structure of Folic Acid

Previous literature states about folate deficiency were associated with increased risk of neural tube defects, various types of cancer, coronary heart disease, Down syndrome etc. Today, FA is delivered orally as oral supplement to patient with these disorders recommended to woman of childbearing age to reduce the risk of neural tube defects. Many countries have implemented mandatory requirement of folic acid in fortification of flour and cereal products with folic acid [14].

Although, there is several methods have been reported the analysis of FA includes, spectroscopic method, flow-injection chemiluminescence, HPLC, LC-MS, capillary electrophoresis and some microbiological assay [11,15, 16].

The present research work was highlights the bioanalytical method development of B complex vitamin folic acid from rat plasma using methylparaben as an internal standard. This work also includes the validation of folic acid bioanalytical method as per international regulatory guidelines. This research could be used as reference information in some bioanalytical method development studies of existing and new drug candidates.

2. RESULTS AND DISCUSSION

2.1 Chromatographic parameters optimization

HPLC has proven to be the best chromatographic system for the detection and separation of compound from the formulations. There are various detectors that are linked with the HPLC system to detect the particular component. Photo diode array ultraviolet detector (DAD) is one of them which are precise tool to identify the compounds. In this research work, UV detector was used for the selection of the suitable wavelength to maximize the signal of the compound whereas, to minimize the signal of plasma compound. For the analysis of the folic acid with internal standard (methylparaben), various composition of mobile phase were tried which are showed in Table 1 and one composition was optimized which give good peak shape with good resolution and stable retention time. Folic acid not has as much solubility in water although it is water soluble vitamin. Trials started with composition of water and methanol but, no peak shape and resolution occurs. Another trial taken with acetonitrile and water composition but same results was obtained as in previous trial. Folic acid is stable at basic pH, so in next trial water phase is replaced with potassium phosphate buffer phase. Methanol is cheap solvent than acetonitrile so, only one trial was taken with acetonitrile and that was replace with methanol. The composition of potassium phosphate buffer and methanol at the ratio of 75: 25 has tried that gives good peak shape with no fronting and tailing and it can be possible because of varying the percentage and pH of potassium buffer and percentage of methanol as organic phase. Finally, potassium phosphate buffer (pH 7.0) Methanol (75:25), chosen as the final mobile phase since it provided the best separation, with higher sensitivity and selectivity for the UV signal of folic acid and methylparaben. This trial was further developed and validated using ICH guideline. The optimized chromatographic conditions of analysis of folic acid and methylparaben are summarized in Table 2.

Table 1. Different Trials of analysis for folic acid and methyl paraben composition

Trials	Different parameters				
	Mobile Phase	Flow rate	Retention time (Min)		
			Folic Acid	Methyl Paraben	
Trial 1	Water: Methanol (50:50)	1 min/ ml	2.8	9.6	Peak shape was not obtained
Trial 2	Water: Acetonitrile (50:50)	1 min/ ml	3.2	10.3	Fronting, Tailing obtained, peak was not proper
Trial 3	potassium phosphate buffer: Methanol (50:50 v/v)	1 min/ ml	5.3	12.5	Peak shape of folic acid not proper but methyl paraben was good
Trial 4	potassium phosphate buffer: Methanol (75:25 v/v)	1 min/ml	6.4	13.2	Good peak shape was obtained with no fronting and tailing

2.2 System suitability

System suitability test is mainly applied to find out the resolution of peak and column efficiency of used HPLC system to verify its ability for analysis of specific component. United State Pharmacopoeia (USP) & International Conference on Harmonisation (ICH) also state that, the system suitability is essential method validation parameter for the chromatographic analysis. System suitability should be performed not only before the experiments but also throughout the whole analytical experiments to behave the selected analytical system properly. The system suitability test when carried out using proposed HPLC method showed that %RSD for peak area and retention time was less than 2 whereas numbers of theoretical plates were more than 2000 for folic acid as well as methylparaben. Form the results it was observed that, the proposed HPLC system is suitable for the analysis. The details of system suitability results are given in Table 3.

Table 2. HPLC optimized conditions for analysis of folic acid

Different parameter	Optimized conditions
Chromatography	Jasco-UV HPLC system
Column	Inertsil ODS C18 column (4.6×100 mm, 5µm)
Mobile phase	potassium phosphate buffer: Methanol (75:25 v/v)
Flow rate	1 ml/min
Total Run Time	20 min
Pressure	88-90 bar
Temperature	40°C
Detection wavelength	283 nm
Retention time Folic acid	6.4
Retention time Methylparaben	13.2

Table 3. Summary of system suitability test for Folic acid & Methylparaben

Parameters	Acceptance criteria	Results			
		Folic acid	% RSD	Methylparaben	% RSD
Retention Time	%RSD ≤ 2%	6.4578	0.0435	13.2145	0.4517
Peak Area	%RSD ≤ 2%	221365	0.0354	146387	0.3841
Theoretical plate	≥ 2000	2503	0.3570	3148	0.0536

(Mean ± SD, n = 3)

2.3 Method Validation

2.3.1 Selectivity

The selectivity is also most fundamental parameter in the method validation typically in the bioanalysis of the compounds. It is also considered as separation selectivity which means, the checking of method weather that is able to separate the known amount of spiked samples with interfering substances. The pre-optimized bioanalytical method to be considered as selective if the analyte present in the sample is completely resolved from the other or the interference do not overlap with analyte.
In this research, described bioanalytical method shows the separation of folic acid (analyte) from the interference (methylparaben). The retention time of folic acid & methylparaben was observed as 6.4 & 13.2 mins respectively. It was observed that, no interfering peaks were present on the same retention time different plasma samples were analyzed. Figure 2, 3 & 4 represents the chromatogram of blank rat plasma sample, standard chromatogram of folic acid in rat plasma & chromatogram of folic acid & methylparaben in rat plasma at different retention time respectively.

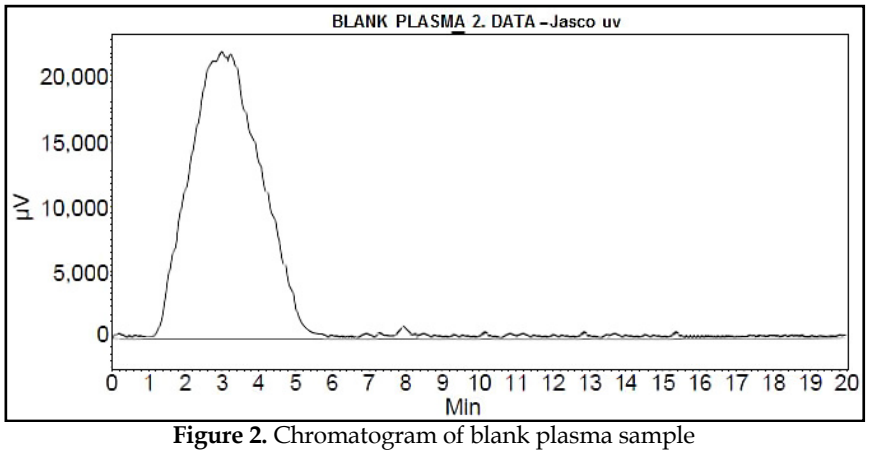

Figure 2. Chromatogram of blank plasma sample

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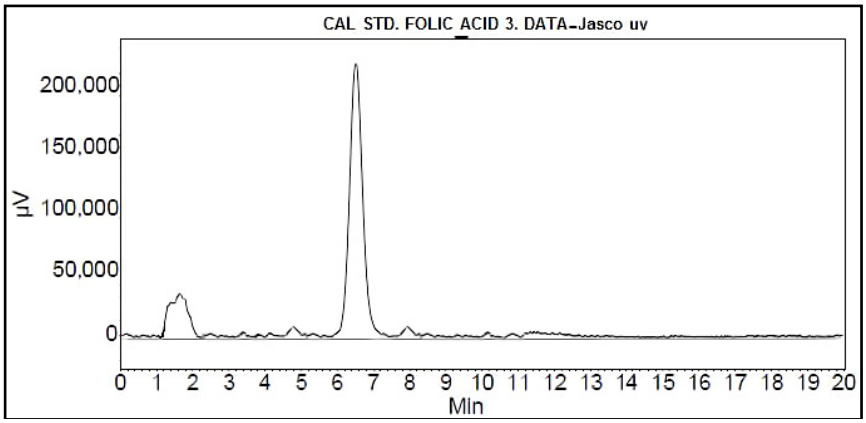

Figure 3. Chromatogram of standard Folic acid

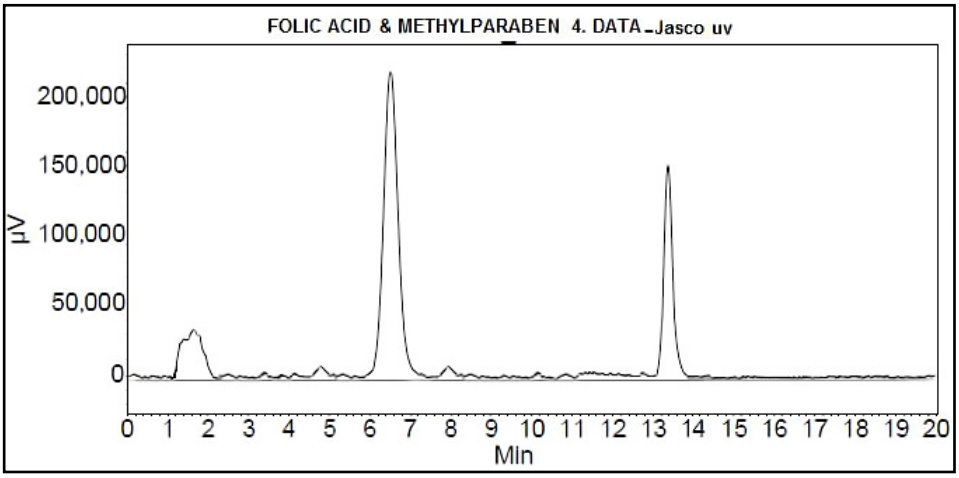

Figure 4. Chromatogram of Folic acid and Methylparaben (IS)

2.3.2 Linearity and Range

Linearity and range are important parameters to validate any bioanalytical method. Linearity is the ability of method to obtain results which is directly proportion to the analyte concentration over different range. Range of bioanalytical method is the interval between the upper and lower concentration of the analyte in the sample. In case of HPLC method development, it is the linear relationship between concentration of analyte and peak area. Considering the importance of linearity & range parameters, six point standard calibrations curve of folic acid ranges from (2-12µg/ml) concentration was developed & depicted in Table 4. Standard curve subjected to least square regression analysis yielded an equation y=35324x+7302.6 for the folic acid with correlation coefficient (r^2) of 0.9992 showed in Figure 5. From the linearity & range study it was conclude that, there is linear relationship between standard concentrations and peak area and also this bioanalytical method has suitable level of linearity, accuracy & precision.

Table 4. Standard solutions with respective area for linearity of folic acid

Folic acid	
Conc. (µg/ml)	**Peak area**
2	75624
4	148264
6	220682
8	296375
10	356281
12	430218

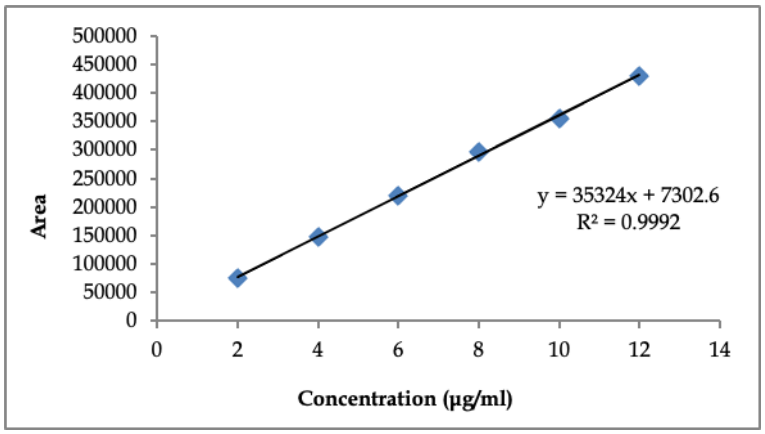

Figure 5. Standard Curve for Folic acid

2.3.3 Accuracy (percentage Recovery)

Accuracy is the closeness of agreement between the values found to the true value. The accuracy studies was performed by determining the percentage recovery of the spiked sample of suitable analyte in to the placebo sample or by comparing the experimental results with the results of certified reference material of known purity. Accuracy data should be developed within its calibration range it could help at any concentration within range to obtain the accurate results. For the folic acid accuracy, recovery studies was performed at at 80, 100 and 120 % by standard addition method, mean recovery of folic acid was found to be 100.30±0.32 whereas, the relative standard deviation (% RSD) was found to be less than 2 was shown in Table 5. From the results obtained by accuracy studies, it was concluded that the proposed method is accurate.

Table 5. Representing Accuracy data of Folic acid

Sample	Spiked level	Theoretical conc. (µg/ml)	Practical conc. (µg/ml)	% Recovery	Mean % Recovery	% RSD
Folic acid	80%	2	2.04	100.87		
	100%	6	5.99	99.82	100.30±0.32	0.530±0.21
	120%	12	12.03	100.21		

(mean ± SD, n = 3)

2.3.4 Precision

Precision is also the fundamental parameter of bioanalytical validation. It expresses the closeness of individual analyte when the analytical method is applied repeatedly from multiple sampling of same homogeneous sample under the prescribed conditions. The precision methods were performed by using intra and inter- day precision. The precision data is generally expressed in the form of % relative standard deviation (%RSD). The pre-optimized bioanalytical method was precise if the % RSD should be less than 2%.

2.3.5 Intra-day precision

Intra-day precision of proposed bioanalytical method was performed by analyzing three different standard solutions (2, 6&12µg/ml) of Folic acid on three different time intervals of the same day. Form the obtained results it was cleared that, the % RSD values of defined concentration were less than 2% which is shown in Table 6.

Table 6. Representing Intra-day precision data of Folic acid

Folic acid			
Nominal Concentration (µg/ml)	**Estimated Concentration (µg/ml)**	**% Assay**	**% RSD**
2	2.0341	101.04	0.5967±0.20
6	6.0120	100.27	0.2548±0.32
12	12.001	99.86	0.0423±0.47
Mean		**100.39**	**0.2979±0.33**

(mean ± SD, n = 3)

2.3.6 Inter-day precision

Inter-day precision study by performed by repeating the standard concentrations (2, 6&12µg/ml) of folic acid on three consecutive days. From the Inter-day precision data it was observed that, the % RSD values are less than 2% which was depicted in Table 7. From the intra & inter-day precision studies it was found that, the proposed bioanalytical method is precise and reproducible.

Table 7. Representing the Inter-day precision data

Folic acid			
Nominal Concentration (µg/ml)	**Estimated Concentration (µg/ml)**	**% Assay**	**% RSD**
2	2.0312	101.12	0.6148±0.36
6	6.0103	100.39	0.2467±0.18
12	12.002	99.96	0.0476±0.56
Mean		**103.82**	**0.3021±0.36**

(mean ± SD, n = 3)

2.3.7 Robustness

Robustness study is performed to identify the critical parameters of the proposed bioanalytical method. In case of HPLC bioanalytical method for the robustness study it is necessary to study the parameters like mobile phase flow rate, column temperature and mobile phase composition which can be varied within particular range. This study was performed by taking the slight change in above parameters. The bioanalytical method is said to be a robust only when, these internal change in parameters did not alter the original results. The results were expressed in terms of % RSD which was less than 2%. From the proposed bioanalytical method it was cleared that, the change in the flow rate of mobile phase, column temperature & mobile phase composition of folic acid did not alter the final results that are obtained previously. The % RSD also found to be less than 2% depicted in Table 8.

Table 8. Representing robustness data of Folic acid

Parameters		**Folic acid**			
		Retention Time	**% RSD**	**Peak area**	**% RSD**
Column Temperature (°C)	35	6.31	0.6238±0.30	221451	0.5427±0.21
	37	6.46	0.3475±0.25	221357	0.0374±0.26
	39	6.45	0.0762±0.28	221521	0.2485±0.32
Mobile phase flow rate (ml/min)	0.8	6.50	0.3674±0.24	221354	0.5384±0.30
	0.9	6.47	0.4917±0.31	221368	0.4681±0.33
	1.1	6.31	0.0362±0.36	221542	0.0365±0.31
Mobile phase composition Buffer: Methanol (% v/v)	74:26	6.42	0.5416±0.32	221674	0.0254±0.26
	72:28	6.49	0.6157±0.28	221219	0.5612±0.32
	71:29	6.51	0.0248±0.27	221456	0.0341±0.21

(mean ± SD, n = 3)

2.3.8 Limit of detection (LOD) and Limit of quantification (LOQ)

The limit of detection (LOD) is also the important parameter for the method validation. It is the lowest amount of analyte present in the sample which is detected by the system but not quantified as exact value. It can be determined by the analysis of known concentration of analyte or by creating the minimum level of concentration at which analyte can be easily detected whereas, limit of quantification (LOQ) is the lowest amount of analyte which can be quantitatively determined with precision and accuracy. It can also establish from visual evaluation same as the LOD. The LOD & LOQ of the proposed bioanalytical method was determined and it was found to be 2.63µg/ml & 10.47µg/ml respectively.

2.4 Stability Studies

Stability study of the analyte in the biological fluid is important in case of bioanalytical method development as this analytical process is directly contact with the blood. This study states about the long term & short-term storage of the analyte. The stability study of proposed bioanalytical method performed to evaluate the stability of folic acid & methylparaben (Internal standard) in aqueous solution as well as plasma sample after exposing to various stress conditions. All stability study was performed under the international regulatory guidelines. Folic acid and methyl paraben stock solution in strength of 1 mg/ml concentration was taken for the study. From the stability study it was observed that, the folic acid & methylparaben in solution remained stable when stored at refrigerator conditions for 7 days and storage at room temperature for 8 hrs. Folic acid and methylparaben was stable in plasma samples when stored at room temperature for 18 hrs. Folic acid was found to be stable for 4 freeze and thaw cycles. Folic acid was also stored in the freezer for 90 days which did not show any degradation.

3. CONCLUSION

A new, relative, simple and reproducible HPLC method for the estimation of Folic acid in rat plasma has been developed and validated. Developed method has simple sample preparation with short analysis time using isocratic elusion. The retention time of folic acid and methylparaben was found to be 6.4 & 13.2 respectively. All the validation parameters were found to be within the acceptance limit. From the stability data of folic acid and methyl paraben samples it was conclude that, this bioanalytical method is was stable and can be used in various pharmaceutical products. This developed method may be applicable for the pharmacokinetic, bioequivalence and drug interaction studies for the estimation of Folic acid in rat plasma. This bioanalytical method gives appropriate data related to Pharmacokinetics, bioequivalence and toxicological studies which can be helpful to complete the dossier that is mandatory requirement to launch any pharmaceutical product in any country. The proposed method was used for the analysis of Folic acid in bulk as well as formulations.

4. MATERIALS AND METHOD

4.1 Chemicals

Folic acid was obtained as gift sample from Micro lab Sikkim India, Methylparaben obtained from TCI chemicals (India) Pvt. Ltd. plasma was collected from the experimental rats. All other chemicals used were analytical grade of Merck.

4.2 Equipments

Chromatographic bioanalysis of Folic acid was performed by using auto sampler Jasco-UV HPLC (AS-2055) quaternary pump system, purified HPLC grade water was used obtained from extrapure water purification system, Ultrasonicator (PCI analytics) was used to sonicate the mobile phase, Vibra HT analytical balance (Essae) was used for weighing purpose.

4.3 Preparation of Solutions

Potassium phosphate buffer was prepared consisting of mixture of 16.282gm/mol of dibasic monohydrogen phosphate & 136.086 gm/mol of monobasic Dihydrogen phosphate in 1L of water and pH

were adjusted to 7.0 with phosphoric acid. Mobile phase was prepared by mixing the methanol and potassium phosphate buffer in the ratio of 25:75 %.

4.4 Preparation of Primary calibration and Internal Standard (IS)

Primary calibration standard Folic acid and methylparaben (Internal standard) (1 mg each) were weighed separately using pre-calibrated weighing balance. These weighed standards were transferred to 1.5 ml of eppendorf tube containing 1 ml of mobile phase. After transferred into the eppendorf tube, it is sonicated for 3 minutes to achieve two different stock solutions (FA-Stock I & Methylparaben- Stock II) of 1mg/ml strength. These eppendorf tubes containing primary standard solutions was packed with aluminum foil and sealed with paraffin film & stored in the refrigerator to avoid the degradation and loss due to the evaporation.

4.5 Preparation of calibration and quality control standards

Previously prepared FA stock-I solution were diluted suitably with selected mobile phase and sonicated to achieve six different calibration standards. By using these calibration standards calibration curve in the range from 2 to12 µg/ml was generated. The calibration standard is 2µg/ml (CAL STD 1), 4µg/ml (CAL STD 2), 6µg/ml (CAL STD 3), 8µg/ml (CAL STD 4), 10µg/ml (CAL STD 5) and 12µg/ml (CAL STD 6). Along these calibration standard three quality control standards (QC) were prepared at the concentration of 2µg/ml (LLQC), 3.6 µg/ml (LQC), 7.2µg/ml (MQC), 9.6µg/ml (HQC) and 1 system suitability (SS) standard (SS STD 6µg/mL) were prepared and used. The prepared calibration and quality control standard were stored in freezer until use. Also, prepared 0.1 ml of methylparaben stock II solution were diluted suitably with mobile in to 1 ml eppendorf and made up the volume with mobile phase itself to achieve the methylparaben stock-III solution strength of 100 µg/ml [18].

4.6 Sample preparation for determination of folic acid in rat plasma

4.6.1 Preparation of folic acid solution/suspension for oral administration

The required quantity folic acid was weighed accurately by using analytical balance and was transferred to glass mortar and pestle. To prepare the suspension of folic acid, sodium carboxy methyl cellulose (Na-CMC) which is 2% of total volume of formulation was added which play the role of suspending agent to give the stability to the suspension. Folic acid & suspending agent was triturated and aqueous fine suspension was prepared by using HPLC grade water.

4.6.2 Procedure for blood samples collection

The protocol for animal experiments was priorly approved by Institutional Animal Ethics Committee of Rajarshi Shahu College of Pharmacy, Buldhana, Maharashtra, India with proposal number (proposal no.1865/PO/Re/S/16/CPCSEA/21-22/P-2). For the blood sample collections, weight of rat was recorded using an animal weighing balance. As per the human dose of folic acid, the accurate dose of folic acid for rat model was calculated which is 0.036 mg (36µg) was administered orally using oral feeding cannula. All time points were of folic acid solution/suspension administered was noted. At each pre-defined time intervals, the blood volume (500 µl) were collected from the rat via retro-orbital plexux using glass capillaries and stored in the heparinized eppendorf. The collected blood was centrifuged at 5000 rpm for 15 min and the plasma was collected. The separated plasma samples (100 µl) were evaluated to get the recovery of folic acid present in the rat plasma. After the recovery the samples were dried and reconstituted in respective 300 µl of selected mobile phase. Mobile phase was filtered through 0.45 µm syringe filter and analyzed for folic acid concentration by HPLC [19,20].

4.6.3 Chromatographic conditions

For the bioanalytical method development of folic acid auto sampler Jasco-UV HPLC system was used with Inertsil ODS C18 column (4.6×100 mm, 5µm) column. Methanol: potassium phosphate buffer (25:75 v/v) mobile phase with total run time of 20 min was developed. Flow rate of mobile phase was adjusted to

1ml/min with injection volume of 10µl at pressure of 100-105 bar and temperature of 40°C. Folic acid was detected at 283 nm as maximum wavelength.

4.7. System Suitability

The system suitability parameter was performed using freshly prepared standard solution (2µg/ml) of folic acid & methylparaben. This solution was repeatedly analyzed using HPLC system. For the system suitability test during analysis, parameters like peak area, retention time and number of theoretical plates were measured and recorded. The acceptance criteria of upper limit of % RSD for peak area and retention time were 2 whereas, acceptance criteria of lower limit of number of theoretical plates was 2000. The system is suitable for analysis or not it depends on these acceptance criteria. System was considered to be suitable only when, the analytical results obtained are within these acceptance criteria.

4.8. Method Validation

The validation part of developed bioanalytical chromatographic method was performed according to the Q2 (R1) guidelines of International Conference on Harmonization (ICH). Validation parameters are plays important role to get the accurate, precise results so, the parameters like selectivity, linearity & range, accuracy & precision, LOD & LOQ, robustness and stability studies were evaluated.

4.8.1 Selectivity

The selectivity parameter was evaluated by taking plasma separated from the blood samples. During the chromatographic analysis, the absence of interfering at the same retention time of folic acid (analyte) and methylparaben (internal standard) was evaluated as proof for the selectivity test.

4.8.2 Linearity & Range

Linearity & range of bioanalytical method of folic acid was calculated by using six different calibration standards. Standard calibration curve was constructed on the basis of these standards representing 2, 4, 6, 8, 10,12µg/mL strength of Folic acid. The linearity graph representing the concentration vs. area was plotted and linearity in terms of R-squared values and respective range were reported.

4.8.3 Accuracy (% Recovery)

Accuracy study of pre-optimized bioanalytical method was calculated using recovery studies by performing the standard addition method. Three levels of percent i.e. 80, 100 and 120 % amount was added externally to the solutions with predefined amount of Folic acid (2, 6 and 12 µg/mL), and the % recovery was calculated.

4.8.4 Precision

The precision data of developed bioanalytical method was evaluated by performing Intra-day and Inter-day studies. Intra-day precision study was carried out by analyzing the three different concentration of folic acid (2,6 and 12 µg/mL) in five replicates whereas, Inter-day precision study was carried out by the analysis of these samples on three consecutive days. The results of both precision studies were obtained in terms of % RSD.

4.8.5 Robustness

The robustness of the proposed bioanalytical method was carried out by doing alterations in flow rate of mobile phase, composition of mobile phase, column temperature and slight change in detector wavelength. Standard solution (6µg/ml) in five replicates analyzed for the retention time and peak area of the folic acid using altered chromatographic conditions. Results of the robustness study were recorded in terms of % RSD. The proposed method was said to be robust only when the % RSD values for both retention time and peak areas were below 2.

4.8.6 Limit of detection (LOD) and Limit of quantification (LOQ)

LOQ of Folic acid is the lowest concentration of folic acid that can be determined with acceptable accuracy and precision. LOD and LOQ were calculated using following formula:
LOD = 3.3 × SD/S
LOQ = 10 × SD/S
Where SD = standard deviation of response (peak area) and S = slope of the calibration curve.

4.9. Stability studies

During the bioanalytical method validation, stability study of folic acid in solution and plasma samples was carried out. Study was proceeding by using two levels of concentrations viz. low-quality control & high-quality control levels which was 3.6& 9.6µg/ml respectively). The long-term stability study was carried out by keeping the plasma samples at freezer and after being stressed to 3 freeze-thawing cycles (24 hours each cycle). Whereas, short term stability study was studied by keeping the quality control standard at 10 °C for 60 hours. These two long term and short term studies compared against the freshly spiked quality control samples [21- 23]

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