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A Thin Layer Chromatography Densitometric Method for Assay of Folic Acid in Tablets

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A High Performance Thin Layer Chromatography (HPTLC) method for analysis of folic acid was developed and validated according to ICH and USP guidelines. The developed method was used for simultaneous qualitative and quantitative analysis of folic acid in tablets. The method was developed using an environmentally friendly mobile phase containing ethyl acetate:methanol:ammonia solution (15:15:0.5 v/v/v) on pre-coated HPTLC silica gel 60 F_{254} glass plates at a detection wavelength of 280 nm using reflectance absorbance and saturation time of 25 min. Densitometric analysis showed two folic acid products being retained at R_f of 0.27 and 0.67 and the area was taken as the sum of the two. The method was specific and no interferences were observed between folic acid peaks and that of the excipients. The calibration curve of folic acid was constructed using both linear and polynomial regression function in the range of 317.19–761.25 ng/spot both with regression coefficient, r^2 of 0.998. The accuracy at nominal concentration of folic acid was found to be 101.05%, % rsd, repeatability and intermediate precision were found to be 1.79% and 1.93 respectively. The developed method is thus simple, accurate, and cost effective with good precision and repeatability for the assay of folic acid in tablets which will be particularly useful in resource constrained countries.

Key words: High performance thin layer chromatography, densitometry, validation, folic acid

INTRODUCTION

Folic acid (also known as folate, vitamin M, vitamin B_9, vitamin B_c or folacin), pteroyl-L-glutamic acid, and pteroyl-L-glutamate are forms of the water-soluble vitamin B_9 [1]. Folic acid is composed of the aromatic pteridine ring linked to para-aminobenzoic acid and one or more glutamate residues and chemically is *N*-(4-{[(2-amino-4-oxo-1,4-dihydro-6-pteridinyl)methyl]amino}benzoyl)-L-glutamic acid (Figure 1) [2].

Folic acid is itself not biologically active, but its biological importance is due to tetrahydrofolate and other derivatives after its conversion to dihydrofolic acid in the liver [3]. The compound is essential for numerous bodily functions. Since humans lack ability for *de novo* biosynthesis of folate, daily folate requirements are met through the diet. The human body requires folate for the synthesis, repair and methylation of DNA, as well as to act as a cofactor in certain biological reactions [4]. It is especially important in aiding rapid cell division and growth, such as in infancy and pregnancy. Children and adults both require folic acid to produce healthy red blood cells and prevent anaemia [5]. Folate deficiency may lead to glossitis, diarrhoea, depression, confusion, anaemia, and congenital neural tube defects and brain defects [6, 7].

Due to the health importance of folic acid in humans, there has been unscrupulous manufacturing and distribution of the compound in the form of tablets which might end to patients resulting to worsening of the condition instead of cure. Thus there is a need of developing a simple, quick and cost effective analytical method to ensure quality products reach consumers. High Performance Thin Layer Chromatography (HPTLC) has increasingly been shown to be a simple, reliable and cost effective method for determination of pharmaceutical products, both in the bulk as well as in single or fixed

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dose combination dosage forms due to increased separation efficiency of the stationary phase compared to thin layer chromatography (TLC) [8-12]. There have been reports of various methods of folic acid analysis in drugs and composite vitamin preparations such as reverse phase high performance liquid chromatography (RP-HPLC) [13-15], HPLC [16], TLC [17-18], adsorptive stripping voltametry at the mercury film electrode [19], tandem liquid chromatography-mass spectrometry [20], ultra-violet (UV) spectroscopy [21], and folic acid analysis in food substances using isotope radio immunoassay and ion-acquisition method [22].

In this study the developed HPTLC method is simple, reliable, efficient and environmentally friendly. Additionally, the method uses small amount of solvents, is time-saving due to short analysis time, and economical by using analytical grade reagents which are not expensive in comparison to HPLC reagents.

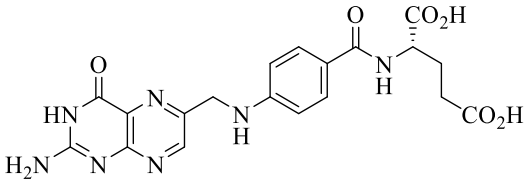

Figure 1: Chemical structure of folic acid.

EXPERIMENTAL

Equipment

TLC scanner 3 operated with Wincats (version 1.4.3), Linomat 5 semi-automatic applicator with a Hamilton syringe of 100 µl capacity, Camag single flat-bottomed developing tank (CAMAG, Muntez, Switzeland) and centrifuge machine (Hermle Z.206A Labortechnik GmbH, Germany) were utilized. Chromatography was carried out on TLC (5 × 10) cm, HPTLC (10 × 10) cm and (20 × 10) cm plates precoated with silica gel 60 F_{254} (Merck, Darmstadt, Germany).

Reference standards and sample tablets

Folic acid reference standard 97 % purity (Sigma Aldrich, Germany) and sample tablets 5 mg label claim (Bensara Pharmaceutical PVT Ltd, Mumbai, India) were used.

Solvents

Analytical grade methanol, ethylacetate and ammonia solution (Carlo ebra reagents, Spain) were used during method development and validation. Distilled water was prepared in-house by reverse osmosis using RO-Purification System (Millipore®, France).

Excipients

Excipients used for simulation were microcrystalline cellulose (FMC BioPolymer, Philadelphia), sodium carboxymethyl cellulose and polyvinyl pyrrolidone cross-linked (Associate Co. Ltd, Shenzhen, China), and magnesium stearate (Shandong LiaochengEhua Medicine Co. Ltd, China).

Analytical procedure

Solution preparation: All samples and reference standard solutions were prepared by dissolving in the diluent (ammonia 13.5 M and methanol, 2:9 v/v) for analysis. The solutions were prepared only when needed.

Standard preparation: A 10 mg of folic acid certified reference standard was weighed into a 10 mL volumetric flask and about 5 ml of diluent added. The mixture was shaken and sonicated for 5 min, the solution made to volume using the diluent, further shaken for 2 min and labeled as folic acid stock standard solution (1 mg/mL). A 1.25 mL of the stock solution was pipetted into a 10 mL volumetric to obtain 0.125 mg/mL of folic acid and the solution made to volume and shaken thoroughly. The flask was labeled as folic acid working standard solution 100% solution.

Sample preparation: Twenty tablets of folic acid were weighed, finely powdered using mortar and pestle and 10 mg of the powder weighed into a 10 mL volumetric flask. About 5ml of diluent was added, the mixture shaken for 1 min, the solution made to volume using the diluent, and then shaken thoroughly. The solution was placed in centrifugation test tubes and centrifuged at 3,000 rpm for 25 min. The contents were filtered and the filtrate labelled as folic acid stock sample solution (1 mg/mL). A 1.25 mL aliquot of the stock solution was pipetted into a 10 mL volumetric flask to obtain 0.125 mg/mL of folic acid and the

volume made up with the diluent and shaken thoroughly. The flask was labelled as folic acid sample solution 100% solution.

Mobile phase: The mobile phase was prepared using 15 mL of ethyl acetate, 15 mL of methanol and 0.5 mL of ammonia solution (13.5 M) which were measured into 50 mL volumetric flask closed and contents were shaken thoroughly for mixing.

RESULTS AND DISCUSSION

Method development

Since the activated HPTLC plates were removed from their packing immediately prior to use there was no need to pre-wash them. Before spotting, the plates were labelled and solvent front marked at 70 mm from the bottom. Five microlitres syringe was loaded at a time with the blank (ammonia 13.5 M and methanol), standard and sample solution and was applied at a distance of 1.5 cm from both side and bottom margins of the plate with an 8 mm band using a Linomat 5 applicator. Application was done in such a way that random errors were minimized by random application of the test concentrations on the plate.

After application the plate was dried in a hot air room before developing. Filter paper was placed to aid saturation at one long side of the developing tank and the mobile phase poured by wetting it and the lid closed for 25 min for tank to saturate before the plate was developed. The scanner wavelength was set equal to 280 nm, slit dimension of 6 mm × 0.45 mm. Scanning of the developed plate was done using reflectance absorbance mode and WinCats (version 1.4.3) planar chromatograph software was used for data acquisition and calculation.

Method validation

A validation protocol was prepared with reference to the ICH Q2 R1 guidelines and USP as reference documents for specificity, linearity, precision and accuracy [23-24].

Specificity and selectivity: Both the solvent and simulated excipients were used during each chromatographic plate run. The densitograms that were obtained during method development was shown to be specific for the assay and was selective for the active pharmaceutical ingredient since there were no blank interferences from the excipients, hence showing that the method was acceptable for assay of folic acid in tablets dosage form (Figure 2).

Linearity*:* Evaluation of linearity for the assay of folic acid was done by preparing five standard concentrations, i.e., 50%, 70%, 80%, 100% and 120% (317.19 – 761.25 ng/spot) of folic acid using serial dilutions from a stock solution. Spots were applied on the plate for each concentration starting with lowest concentration to avoid carryover effect. The procedure was repeated for three days. The results were analyzed using the peak areas of the developed densitograms [23]. Both the linear (r^2 = 0.998) and polynomial (r^2 = 0.998) regression function was evaluated and in both case had r^2 >0.98 (Table 1) hence linear regression was selected for use during accuracy testing.

Table 1: The linear and polynomial regression equations obtained from the HPTLC densitometric method for folic acid reference standard

Parameter	Polynomial regression	Linear regression
Concentration range (ng/spot)	317.19 – 761.25	317.19 – 761.25
x^2 -coefficient	-0.0014	-
Slope	12.81	11.24
y-intercept	2025	2418
r^2	0.998	0.998
n	3	3

n = number of days

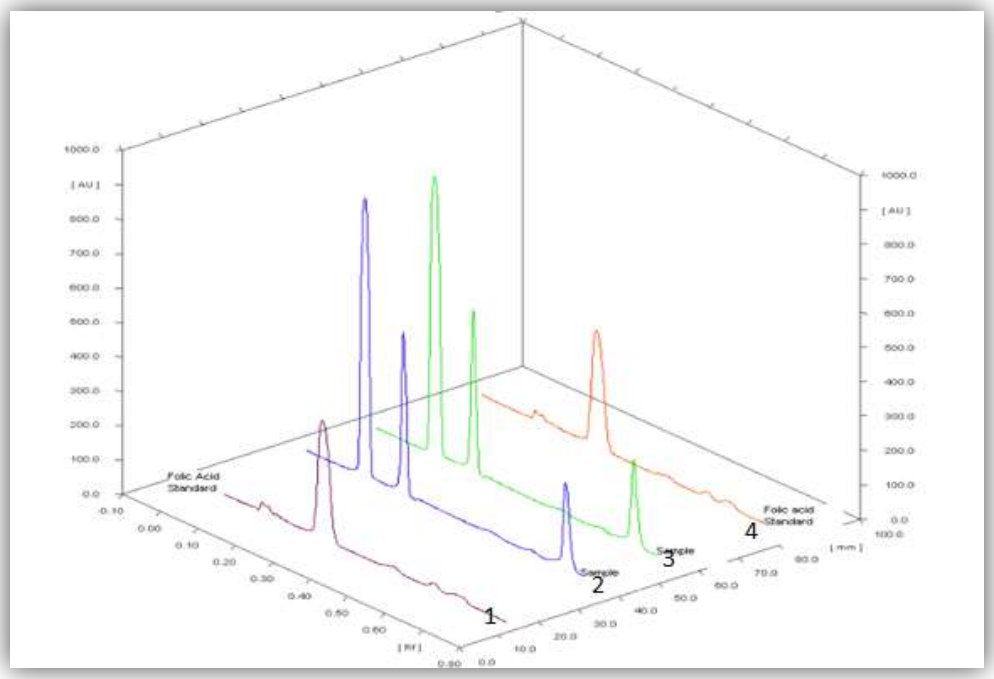

Figure 2: Typical densitograms pattern showing method specificity of solution containing folic acid reference standard and sample solution using a mobile phase containing ethyl acetate: methanol: ammonia (15:15:0.5 v/v), saturation time of 25 min using 10 cm x 10 cm HPTLC plate and 5 µL application volume at a detection wavelength of 280 nm. Folic acid (0.125 mg/mL) R_f 0.27 and 0.67. Track 1 and 4: Folic acid reference standard; Track 2 and 3: folic acid sample tablet.

Precision: Repeatability and intermediate precision were done for the assay method using sample tablets in which six replicate sample solutions were prepared independently corresponding to 100% level of the assay concentration. Intermediate precision was done by using two analysts in different days. The calculated percentage relative deviation (% rsd) by using peak areas were found to be 1.79% and 1.93% for repeatability and intermediate precision respectively hence complied with the ICH guideline (Tables 2 and 3).

Table 2: Repeatability of folic acid sample tablet

Parameter	Sample repeatability
% Concentration	100
Mean	6370.61
Sd	114.51
% rsd	1.79

n = 6 (number of replicate samples)

Accuracy: Accuracy was evaluated by determination of percentage of folic acid reference standard spotted as controls by using a calibration curve at 80%, 100% and 120% of the assay concentrations. The controls were weighed in triplicate for each concentration. Each concentration was spotted on a separate plate in triplicate. Assay of sample tablet was done where percentage assay at 80%, 100% and 120% was determined using the calibration curve. The accuracy was within the limits according to USP [23] for folic acid reference standard (range of 98% to 102%) for all concentrations in which it ranged from 99.92% for 80%, 101.05% for 100% and 101.02% for 120% (Table 4). Assay of folic acid tablets was carried out using the developed method and was found to be within the USP limits of 95% to 105% (Table 5).

Table 3*:* Intermediate precision for folic acid tablets

Parameter	Analyst 1	Analyst 2	Average
Mean	6370.61	6458.00	6414.31
Sd	114.51	126.64	123.83
% rsd	1.79	1.96	1.93

n = 6

Table 4: Accuracy values for folic acid tablets

Concentration levels	80%	100%	120%
% Recovery	99.92	101.05	101.02
Sd	10.35	7.55	1.80
% rsd	2.07	1.20	0.24

n = 9

Table 5: Assay values for folic acid tablet at various concentration using the developed method

Folic acid (5)mg	Expected amount (ng)	Amount found (ng)	Percentage
120%	750.00	757.62	101.05
100%	625.00	631.56	101.02
80%	500.00	499.58	99.92

n = 3

CONCLUSION

A facile and robust chromatographic technique for assay of compounds was developed and validated according to ICH and USP guidelines [23–24]. Compared to previously reported methods [13-16, 19-22] the developed method is simple, accurate, and cost effective for routine analysis of folic acid tablets using an environmentally-friendly mobile phase containing ethyl acetate:methanol:ammonia solution (15:15:0.5 v/v/v).

Good selectivity and an acceptable level of specificity were observed together with % rsd below 2% for both repeatability and intermediate precision. Additionally, the results obtained after assay of the sample tablets met the USP acceptance criteria of being between 95% and 105% of the label claim which shows that the method is suitable for quantification of folic acid and renders it useful for routine screening and analysis especially in resource constrained countries.

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