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High-Performance Liquid Chromatographic Determination of Folic Acid Content in Pharmaceutical Formulations marketed in Southwest Nigeria.

Olufunmilayo Ebunoluwa Adejumo*[1], Fatimah Atinuke Badmus[1], Morenike Abosede Oluboba[1], Oluyemisi Adebowale Bamiro[2]

[1]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmacy, Olabisi Onabanjo University, Sagamu, Ogun State, Nigeria.
[2]Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmacy, Olabisi Onabanjo University, Sagamu, Ogun State, Nigeria.

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* *Corresponding Author:*
Email: funmijumo85@gmail.com
+234 8132512825
https://orcid.org/0000-0003-4487-3957

ABSTRACT

Background Folic acid is presented as tablets and syrups or incorporated into food, drinks, and beverages frequently for fortification as a remedy to folic acid deficiency which may cause severe health consequences. Consequently, the analysis of folate in pharmaceutical preparations by quantitative methods like high-performance liquid chromatographic (HPLC) in this study cannot be overemphasized.
Methods: The parameters investigated included weight uniformity, hardness, friability, and disintegration rate. A validated, reversed-phase HPLC (RP-HPLC) method ran with trifluoroacetic acid and acetonitrile (90:10) as mobile phase, on a C18 column, at 40°C, was used to assay the content. The method employed a flow rate of 1.0 mL/min and a wavelength of 290 nm. The injection volume was 10 µL.
Results: The weight of the tablets ranged between 9.86±0.01-32.30±0.01 mg, hardness was 7.52±0.80-15.48±1.53 kg/cm^3, and tablets' friability was less than 1%. All the tablets passed the disintegration test for uncoated tablets except FAJ. Folic acid was eluted at 2.616 min and the RP-HPLC method displayed good linearity over the concentration range of 5-200 ppm, with a correlation coefficient (r^2) of 0.9998. The relative standard deviation (% RSD) for precision was 0.2%, while the LOD and LOQ values were 1.3191 and 4.3969 µg/ml respectively. The % RSD for reproducibility was < 5% in the assay of folic acid tablet samples. Only 50% of the brands passed the assay of content i.e., fell within the specified range of not less than 90 % and not more than 110 % according to reference specification.
Conclusion: None of the brands of folic acid tablets evaluated complied with all the specifications for each test carried out whether compendia or non-compendia test. Hence, stringent quality control including adequate storage control should be applied for the release of such vitamin preparations.

1. Introduction

Vitamins are essential nutrients that must be provided to the body regularly in minute amounts to perform various chemical and physiological functions[1]. They are groups of organic compounds that are essential for protection, metabolism, health maintenance, normal growth, and optimal nutrition. They are required in small amounts in the diet because they cannot be synthesized by humans.[2] Hence, must be obtained from outside sources like diet, rumen of bacteria, and sun.

The WHO has reported frequently that an estimate of about

2 billion people globally is at risk of micronutrient deficiencies (vitamins, minerals, and trace elements).[3] This is in addition to the estimation by 5 UN agencies that 821 million people globally are undernourished, which predisposes them to the risk of vitamin and essential nutrient deficiencies.[4] The International Body defines vitamins as essential nutrients for optimizing health and, consequently, life; especially for those stages of life that present with a higher risk of deficiency than others i.e., pregnant women, infants, children, adolescents, and the elderly.[3,5]

Vitamin B9 commonly known as folic acid and chemically as pteroyl-L-glutamic acid (Figure 1) is a synthetic, water-soluble folate that is usually obtained from pharmaceutical preparations due to inadequate sourcing from diet. Folate which is the deprotonated ion of folic acid the neutral molecule, co-exist in water. Folate is converted by the liver to N5–methyltetrahydrofolate, a major circulating form in blood, that is responsible for the one-carbon transfer enzymatic reactions (homocysteine to yield methionine). Following conversion, vitamin B9 or folate becomes tetrahydrofolate, its active form. It is an essential molecule in the synthesis of nucleic acids (DNA and RNA). This is especially important during periods of rapid cell division and growth such as infancy and pregnancy.[6] Neural tube defects are common in children whose mothers were deficient in folic acid during pregnancy,[7] thus, pregnant women should receive folic acid supplementations as a preventive approach.[8] This is because, in pregnancy, there is an increased risk of foetal neural tube defects (NTDs); hence, pregnant women are at risk of folate deficiency because folate requirement is significantly increased during pregnancy, especially during periods of rapid foetal growth.[8] Thus, folate deficiency hinders DNA synthesis and cell division affecting most notably, bone marrow and cancer, both of which participate in rapid cell division.[9,10]

Folate deficiency can also cause megaloblastic anaemia, a type of macrocytic anaemia, and prompts a differential diagnosis with vitamin B12 deficiency, which also causes megaloblastic anaemia.[11] Methylenetetrahydrofolate is involved in the synthesis of thymidine, an important component of the DNA. Methyl vitamin B12 mediates the reaction in which the amino acid methionine, which is required for the synthesis of myelin, is generated from homocysteine. During this process, methyl tetrahydrofolate is also converted to tetrahydrofolate. The normal generation of methyl tetrahydrofolate depends upon an adequate supply of both folic acid and vitamin B12. Deficiency of either of them can produce a defect in all the tissues with the rapid rate of cellular proliferation, e.g., the bone marrow (resulting in megaloblastic anaemia) and the gastrointestinal tract.[12]

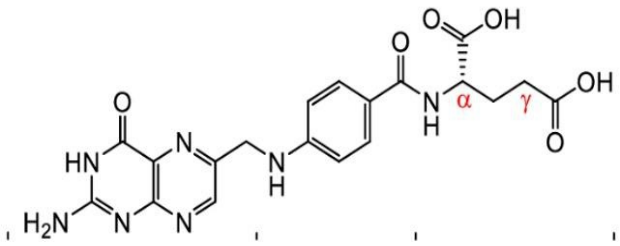

Figure 1: Chemical structure of folic acid (Vitamin B9)

Chemical equivalence is a concept that establishes the significance of the content of food products generally and vitamins, to their desired effect on the human body or more specifically, their bioavailability[13]. This is because substandard pharmaceutical products have a deleterious effect at all levels of health care delivery. Therefore, two chemically equivalent pharmaceutical products such as vitamins in this case are those that have identical amounts of active ingredients, identical strength, identical dosage forms, and meet existing physicochemical standards in the officials' compendia.[14] The standardization of the chemical equivalence of vitamins is of great importance because these vitamins play vital roles in human metabolic processes.

Folic acid can be determined by many methods including colorimetric, potentiometric, and spectrophotometric measurement of the extinction maxima of the UV absorption curve; and by the spectrometric measurement after oxidative fission of folic acid to 4-aminobenzoyl-glutamic acid followed by diazotization and coupling to give an azo dye. Other studies have also reported volumetric and spectrometric assays for folic acid,[15] as well as conventional chromatographic procedures such as thin-layer column chromatography (TLC) and high-performance liquid chromatography (HPLC).[16] Other methods reported for folic acid quantitation in tablets include FTIR spectroscopy, isotope-dilution liquid chromatography/tandem mass spectrometry (LC/MS/MS),[17] and Liquid Chromatography-Tandem Mass Spectrometry.[18] Nevertheless, the Brazilian Pharmacopoeia,[19] recommends high-performance liquid chromatography (HPLC) for the identification and quantification of FA in pharmaceutical formulations.

The HPLC method has gained more preference because of its abundant advantages; more rapid, accurate, sensitive,

and selective assay yielding a better resolution.[20] The method 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.[21] Measurement of drug concentrations is important, especially in clinical toxicology, which either fulfils research purposes or clinical purposes. The aim of this study was to determine the actual versus labelled concentrations of folic acid in selected pharmaceutical formulations available in the Nigerian market by HPLC.

1. Materials And Methods

2.1 Materials

The materials used were Emsure HPLC Grade Acetonitrile (>99.9%), CDH Trifluoroacetic Acid (TFA) for synthesis (>99%), Losa Chemie Sodium hydroxide pellets (97%) Extra pure, Distilled water, Folic Acid Standard (Sigma Aldrich, purity 99.8%).

2.2 Method

2.2.1 Determination of uniformity of weight

Twenty (20) tablets from each brand were randomly selected and weighed using Adventurer-pro Analytical Balance. The average tablet weight from each brand was determined and the percentage and standard deviation were calculated. Determination was done in triplicate.[22]

2.2.2 Determination of Tablet Hardness

Three tablets were selected from each brand, each tablet was placed between a fixed and movable jaw of a Ketan hardness tester, and force was applied through a screwdriver spring by turning the screw. The average force needed to break the three tablets from each brand (in kg/cm) was calculated.[22]

2.2.3 Determination of Tablet Friability

Ten (10) tablets from each of the brands were weighed (W_1) and placed in a friabilator, operated at 25 revolutions per minute for 4 minutes (100 revolutions). The tablets were removed after friabilation, dusted, and re-weighed (W_2). Determination was done in triplicate. The result of the test was expressed as percentage weight loss using the equation:

$$\% \, friability = \frac{W_1 - W_2}{W} \quad \text{.....Equation 1}$$

2.2.4 Determination of Disintegration Time

The disintegration test was carried out in distilled water at a temperature of 37±0.5^{0}C in a disintegration apparatus. Determination was done in triplicate.

2.2.5 Chromatographic Conditions

Chromatography was performed on a Waters X-bridge C18 (100 x 4.6 mm, 3.5 µm) column. The mobile phase was made up of HPLC grade 0.1%v/v trifluoroacetic acid (90%v/v) and acetonitrile (10%v/v) at a ratio of 90:10, which was filtered and degassed using a vacuum pump before use. All analyses were run at a flow rate of 1.0 ml/min at a temperature of 40°C. Detection was made at a wavelength of 290 nm. The injection volume was 10 µL.

2.2.6 Preparation of standard solutions of folic acid

Folic acid (25.1 mg) standard (99.98% purity) was weighed into a 25 mL volumetric flask covered with aluminium foil to keep away sunlight, 0.1N NaOH (5 ml) was added and shaken to completely dissolve the folic acid. It was then made up to mark with water to get a stock solution of 1 mg/ml (1000 ppm). Graded concentrations of folic acid ranging from 5 ppm-200 ppm were prepared from the stock solution. The graded concentrations were injected separately to generate the standard calibration curve (Figure 2).

2.2.7 HPLC Method Validation

2.2.7.1 Linearity

The linearity was studied from the standard concentrations ranging from 5 µg/ml to 200 µg/ml. The calibration curve of peak intensity was plotted against concentration and the correlation coefficient was determined.

2.2.7.2 Precision

The precision was determined by injecting 5 µg/ml of folic acid three times and the percentage relative standard deviation (%RSD) of peak areas was calculated.

2.2.7.3 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection can be defined as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified under the stated conditions of the test, while the limit of quantification can be defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated conditions of the test. These were

determined from the standard deviation of the peak response and the slope of the calibration curve.[23]

2.2.8 Assay of the active ingredient in folic acid by HPLC

2.2.8.1. Preparation of Local Brands

Forty (40) tablets of folic acid containing 5 mg of the active ingredient were pulverized. The equivalent of 100 mg folic acid was weighed and transferred into a 100 mL volumetric flask. 0.1 M NaOH (30 mL) was added and sonicated for 10 mins after which it was made up to 100 mL mark with water to obtain a stock solution of 1 mg/mL. It was sonicated for 10 mins using an ultrasonic bath and filtered using a 0.45 µm acrodisc syringe filter and ready for HPLC analysis.

2.2.8.2 Preparation of Foreign Brands

Forty (40) tablets of folic acid containing 0.8 mg of the active ingredient were pulverized. The equivalent of 8 mg folic acid was weighed and transferred into a 100 mL volumetric flask. 0.1 M NaOH (30 mL) was added and sonicated for 10 mins after which it was made up to 100 mL mark with water to obtain a stock solution of 0.08 mg/mL. It was sonicated for 10 mins using an ultrasonic bath and filtered using a 0.45 µm syringe filter and ready for HPLC analysis.

2.3 Statistical Analysis: All results were obtained in triplicates, and they were subjected to statistical analysis of mean and standard deviation, using Microsoft Excel 2016 version[24.]

3. Results

Six brands of folic acid (with structure in Figure 1) tablets were investigated, and all were within their shelf life with registered NAFDAC numbers except brand FAN which was without a NAFDAC number (Table 1).

Table 1: Brands of folic acid with batch number, manufactured, and expiry date.

Sample	Country	Batch number	Manufacturing date	Expiry date	NAFDAC number
FAE	Nigeria	2459A	7/21	7/24	04-0268
FAM	Nigeria	10602221	5/21	4/24	04-2511
FAJ	Nigeria	21063101	6/21	5/24	B4-1985
FAR	Nigeria	2101	04/21	3/24	A4-2658
FAO	USA	OJ924002	9/21	2/23	A11-0360
FAN	USA	22763		4/23	

The physicochemical parameters of the various brands are presented in Table 2. All the brands passed the weight uniformity test except brand FAJ with a deviation >10%. The hardness of the tablets ranged between 7.52 to 15.48 kg/cm^3. The BP recommends a hardness of 5-8 kg/cm^3 for tablets.[25] Friability value not greater than 1% is acceptable.[25] All the brands passed the friability test. All folic acid brands passed the disintegration time test, meeting the pharmacopeia specified 15 minutes of uncoated tablets,[25] except FAJ.

Table 2: Properties of Folic acid samples

Sample code	Uniformity of weight(mg)	Hardness (Kg/cm^3)	Friability (%)	Disintegration time (min)	Assay Concentration (%)
FAE	11.08±0.16	7.66±0.59	0.92±0.67	2.53±0.25	102.64±0.26
FAJ	14.02±0.01	7.52±0.80	0.92±0.15	22.17±0.12	105.03±4.39
FAN	19.55±0.01	8.38±0.41	0.30±0.21	4.45±0.55	131.85±0.09
FAM	11.67±0.01	15.48±1.53	0.38±0.17	0.83±0.07	112.12±2.17
FAO	32.30±0.01	8.69±0.79	0.02±0.01	0.77±0.26	107.48±0.99
FAR	9.86±0.01	8.11±0.40	0.48±0.11	1.46±0.39	162.36±0.83

The HPLC method was validated and found to be suitable for the determination of FA content in this tablet dosage form. Quantitation was done with a calibration curve (generated from standard concentrations of folic acid) which had a regression coefficient of 0.998 indicating good linearity at a concentration range of 5 – 200 µg/ml. The calibration curve is depicted in Figure 2.

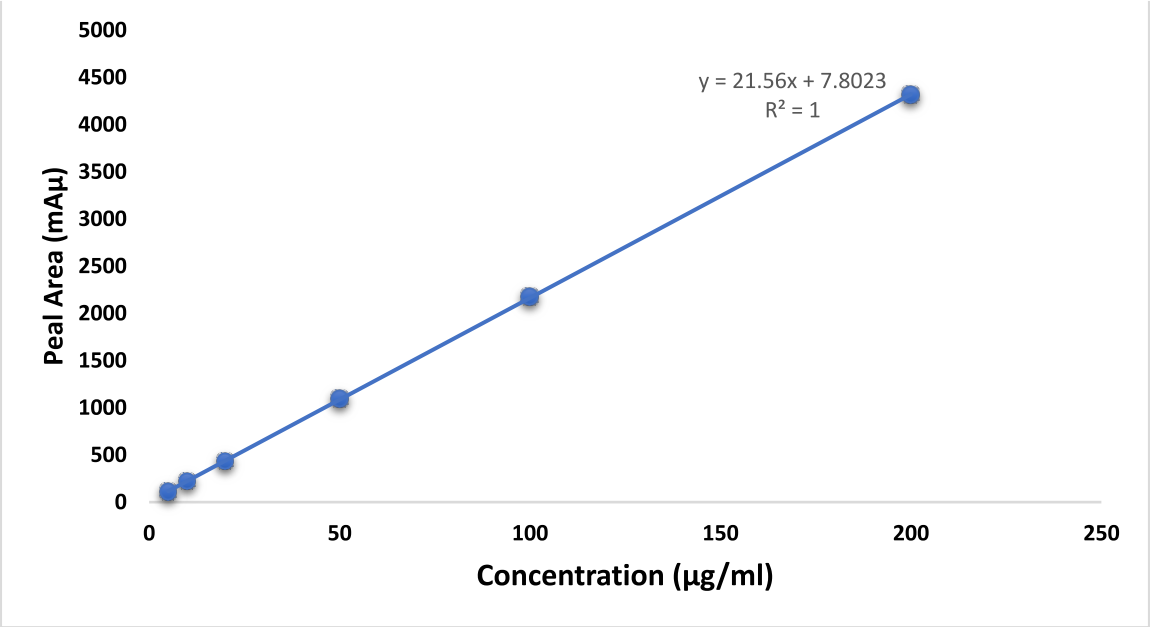

Figure 2: Calibration plot of standard folic acid

The %RSD for precision and retention time was < 2%, while LOD and LOQ were 1.3191 µg/mL and 4.3969 µg/mL respectively. The procedure detected the lowest level of concentration (µg/mL) of analyte in the samples. The results for validation parameters are given in Table 3.

Table 3: Validation result of HPLC

Linearity Range	5-200ppm
Correlation (r^2)	0.998
Retention time precision (n=3)	0.4208 %RSD
Area precision (n=3)	0.0382 %RSD
Limit of detection (LOD)	1.3191 µg/ml
Limit of quantitation (LOQ)	4.3970 µg/ml
Reproducibility	0.9256 D

4. Discussion

Six brands of folic acid tablets were investigated with four brands from Nigeria and two from the United States of America, all marketed in Nigeria and in possession of the regulatory agencies number. The weight uniformity test is a compendia test used to detect the variation in the weight of each tablet from the batch. The significance of uniformity of weight is to verify that the tablet in the batch falls within the appropriate size range and conforms to the amount stipulated. According to the BP[25] for tablets having a mean weight of less than 80mg, not more than two tablets are permitted to deviate from the mean by ±10%, and none of the tablets must have a percentage deviation of more than double the relevant percentage deviation. Weight uniformity is important as variation in weight can affect content uniformity or the amount of drug in the tablet.[26] The hardness or crushing strength of a tablet is the load or force in kg required to crush the tablet when placed on its surface. The resistance of tablets to capping, abrasion, or breakage during storage, handling, and transportation before usage depends on their hardness.

Generally, all the brands were within this range except brand FAM. Tablet hardness has been shown to affect the disintegration of tablets as high hardness will increase disintegration time and this would influence the dissolution of the tablet. Surprisingly, this same brand FAJ had the lowest hardness, and it would have been expected to have the lowest disintegration time. This could have been due to the type of excipient used by the different manufacturers. Friability is the ability of a tablet to withstand stress during packaging, transportation, and handling. All the brands passed the friability test with acceptable values of not greater than 1%.[25] Tablet disintegration is an important physicochemical parameter in solid dosage forms as it is known to be a rate-limiting step in drug absorption. The pharmacopeia specified 15 minutes for the disintegration of uncoated tablets,[25] was met by all the folic acid tablet brands except sample FAJ.

The aim of the assay is to ensure the presence of the required amount of the active ingredient. The significant variation could lead to ineffective therapeutic drug levels or overdosing that may lead to toxicity.[26,27] Equally, acute, or chronic ingestion of a large dose of folic acid generally manifests as neurological complications, which are reversible[27]. According to USP,[28] the folic acid content range is between 90-110%. Consequently, only 50% of the brands (FAE, FAJ, and FAO) passed this test. There was a significant ($p<0.05$) variation in the content assay of the tablets. This showed that the HPLC method used for the assay was linear, precise, sensitive, and reproducible for the determination of folic acid content in tablet pharmaceutical preparations.

An industrial pharmacist usually encounters several problems during manufacturing which may include visual defects and functional defects. However, an ideal tablet should be free from any visual or functional defects. Tablet processing problems can arise from formulation issues or

problems encountered in the compression equipment, or both, and these problems have not decreased despite the advancements and innovations in tablet manufacture.[29,30] However, for whatever reason the tablets are made, they must conform to those established standards of elegance and accuracy expected from medicines dispensed to patrons. Hence, the samples that failed the expected folate content as labelled could constitute a significant lowering of the DFEs (Dietary folate equivalent) recommendation for all age groups and a considerable health risk, particularly, to pregnant women who require the drug to prevent infants born with neural tube defects. The Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO) Expert Consultation report in 1988[31] enumerated three states of folate nutrition: folate adequacy, impending folate deficiency, and overt folate deficiency. Folate supplementation has also been reported to improve seizure control in children taking long-term antiepileptic drugs;[32] but grave consequences as earlier described attend folate insufficiency including megaloblastic anaemia.[33,34.]

We observed that two samples, FAN and FAR had folic acid content that fell within this high-level range of 130% and above. However, folic acid being a water-soluble vitamin, would dispose of any excess intake, and usually to be easily excreted in urine.[3] Although oral folic acid is generally regarded as safe and not toxic for normal humans, it may cause neurological injury when given to patients with undiagnosed pernicious anaemia.[35] Hence, the control of the quality and the content of folic acid in formulations cannot be overemphasized; particularly for such an important dietary nutrient whose deficiency in pregnancy causes neural tube defects in children born to such pregnant women.[7] The variation that existed between the different brands of folic acid in this study may be due to differences in the excipients, method of manufacturing, or other problems during manufacturing, in addition to compression force and equipment.[29] The HPLC method, which was linear, precise, sensitive, and reproducible can be applied to determine folic acid concentrations in pharmaceutical preparations.

5. Conclusion

None of the brands of folic acid tablets evaluated complied with all the specifications for each test carried out whether compendia or non-compendia test. Hence, stringent quality control including adequate storage control should be applied for the release of such vitamin preparations. The folic acid samples used in the study may have equally degraded due to poor storage conditions.

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