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COMPARATIVE IN VITRO EVALUATION OF (6S)-5-METHYLTETRAHYDROFOLATE BRANDS: THE POST MARKET SURVEILLANCE FOR QUALITY ASSESSMENT

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ABSTRACT

The biologically active form of folate, chemically referred to as (6S)-5-methyltetrahydrofolic acid (5-MTHF) may be a superior alternative to folic acid for treating mental health disorders and improving folate levels. However, as L-5-MTHF is susceptible to thermal degradation, it is necessary to assess its stability parameters to ensure that a branded supplement is efficacious and provides the desired dosage. Three different brands of L-5-methyltetrahydrofolate specified as: Metafolin® (registered by Merck KGaA), Quatrefolic® (registered by Gnosis) and Cerebrofolate™ (registered by MTHFR Solutions, LLC) as well as a generic amorphous calcium salt form of L-5-MTHF were selected for analysis by appearance, content assay, total impurities, water content and free folate levels. USP-NF standards were followed for the various tests. The present study concluded that Metafolin® and Cerebrofolate™ exhibited superior stability and potency in comparison to the amorphous salt and glucosamine salt forms. It is imperative that manufacturers of L-5-MTHF ensure their final product meets all quality control criteria and remains therapeutically active over time.

Keywords: L-methylfolate, stability, in vitro evaluation, assay, quality, Free Folate, 5-MTHF

INTRODUCTION

Folate is a water-soluble B-vitamin essential for the synthesis of DNA and methylation pathways. The various forms of folate share a common chemical structure which differs by the number of glutamic acid groups, the presence of carbon substituents at N5 and N10 positions, and the presence of either an oxidized or reduced pteridine ring **[1-4]**. Dietary folate (from food), folic acid, and L-Methylfolate are the main folate sources available for human consumption. The specific structural arrangements of different folates affect their bioavailability and metabolism. Dietary folates are only about 50% bioavailable, as they must be hydrolyzed by a brush-border hydrolase during digestion in the gut. Folic acid is the synthetic form of folate and the most stable form, as it is the oxidized state and has only one glutamate residue. Folic acid (FA) is therefore utilized in most supplements and dietary fortification. However, the administration of folic acid is not well suited for individuals with MTHFR gene variants which affect approximately 40% of the general population **[5]**. L-Methylfolate is a reduced form of FA which functions as a cofactor in the transfer and utilization of one-carbon groups; it provides one-carbon groups for the biosynthesis of purines, pyrimidines, and DNA, and is also essential for methionine regeneration. All types of folates in the body are eventually metabolized to become 5-methyltetrahydrofolate (5-MTHF). Unlike dietary folates, folic acid must first undergo a four-step process via the enzyme dihydrofolate reductase (DHFR) to be converted to tetrahydrofolate (THF). Tetrahydrofolate then becomes 5,10-methylene THF before methylenetetrahydrofolate reductase (MTHFR) is able to methylate 10,5 Methylene-THF to become 5-MTHF **[2]**.

Currently, several nutritional supplements contain calcium salt-based (6S)-5-methyltetrahydrofolic acid (5-MTHF or methylfolate), a natural form of folate. This form can enter the bloodstream directly and be utilized by the body without requiring reduction by DHFR or MTHFR **[6]**. Various randomized clinical trials have demonstrated methyltetrahydrofolate to be as effective as folic acid at increasing concentrations of blood folate necessary for birth defect prevention. Additionally, supplementation with 5-MTHF has been demonstrated to improve low levels of 5-MTHF synthesis caused by genetic variations in the genes for folate metabolism or FA-induced enzyme inhibitions. It has also been found that 5-MTHF has a greater therapeutic potential than FA in the prevention and potential treatment of mental health conditions, including perinatal depression which supports switching from FA to 5-MTHF during pregnancy **[2, 7]**. Other clinical studies have also demonstrated that (6S)-5-MTHF has antioxidant potential **[8]**.

Oral absorption and bioavailability of folic acid is low for a range of clinical conditions. To address this, the crystalline form (6S)-5-MTHF calcium salt and amorphous form (6S)-5-MTHF glucosamine salt have been introduced **[9]**. When exposed to high temperatures, air, light, low pH, and the presence of reducing agents, all folate compounds can degrade **[10]**. 5-MTHF is more susceptible to thermal degradation than folic acid, and therefore it is necessary to assess the stability parameters to ensure the final concentration of 5-MTHF is in the desired dosage form & range.

The marketed brands of crystalline (6S)-5-MTHF calcium salt include patented brand Metafolin® (registered by Merck KGaA) and trademarked brand Cerebrofolate™ (registered by MTHFR Solutions, LLC) whereas patented brand Quatrefolic® (registered by Gnosis) contains an amorphous glucosamine salt form of (6S)-5-MTHF. Therefore, the current study was designed to evaluate and compare the aforementioned brands of L-5-methyltetrahydrofolate as both calcium and glucosamine salts in terms of their stability, as well as their changes in appearance at room temperature, content assay, total impurities, water content, and claimed potency regarding free folate levels.

MATERIALS AND METHODS

Materials

Three different brands of L-5-methyltetrahydrofolate and one generic amorphous form of L-5-MTHF Calcium were selected for analysis via appearance, content assay, total impurities, water content and Free Folate levels. The brands were labelled as Metafolin® (Sample 1), Quatrefolic® (Sample 2) and Cerebrofolate™ (Sample 3). An amorphous calcium salt form of (6S)-5-methyltetrahydrofolate was also used (Sample 4). USP-NF standards were followed for the various tests **[11]**.

Ethical Approval

Ethical approval was obtained for this study by the Ethical Review Board of Hamdard University, Islamabad, Pakistan (ERB/HUIC/358).

Stability and Appearance at Room Temperature

The physical stability and changes in appearance were determined by exposing the four samples to open air room temperature at 25 degrees Celsius and relative humidity 65% for 90 days. The changes in physical appearance were recorded on days 0, 15, 30, 45, 60 and 90.

The x-ray diffraction results were also compared for Sample 3 with the patented brand (sample 1) to confirm the presence of Type I Crystalline (6S)-5-MTHF calcium salt which has the highest stability in sample 3 **[12]**.

Content Assay

Content analysis using HPLC for all 4 of the samples was performed on days 0, 15, 30, 45, 60, and 90. Buffer solution comprising sodium dihydrogen phosphate dihydrate in water with a concentration of 7.8 g/L was prepared. Two solutions were prepared using the following method: For Solution A, the buffer was adjusted to pH 6.5 with a solution of sodium hydroxide with concentration of 32% w/v. For Solution B, a solution was prepared using Methanol and Buffer in a ratio of 35:65. The solution was adjusted to pH 8 using 32% w/v sodium hydroxide solution. The standard solution used was USP Calcium DL-5-Methyltetrahydrofolate RS in water with a concentration of 0.5 mg/mL whereas solutions of 0.5 mg/mL concentration of Sample 1, 2, 3 and 4 were prepared. Solution A and B through gradient elution were used as mobile phase in the column as described in **Table 1**.

Table 1: Gradient Elution for Mobile Phase

Time (min)	Solution A (%)	Solution B (%)
0	100	0
14	45	55
17	0	100
24	0	100
24.01	100	0
33	100	0

The percentage content of calcium 5-methyltetrahydrofolate ($C_{20}H_{23}CaN_7O_6$) was calculated as follows:

Result= $(r_u / r_s) \times (C_s / C_u) \times 100$

where,

r_u = Sample solution peak response

r_s = Standard solution peak response

C_s = Standard solution concentration of nutrient (mg/mL)

C_u = Sample solution concentration of nutrient (mg/mL)

The acceptance criteria for content assay is 95.0%–102.0%.

Total Impurities

To determine the total impurities in the four samples on days 0, 15, 30, 45, 60, and 90, the same procedure was followed as described in the content assay. The following equation was used to determine the percentage of impurities in all samples:

Result = $(r_u / r_s) \times (C_s / C_u) \times F \times (M_{r1} / M_{r2}) \times 100$

r_u = *Sample solution* impurity peak response

r_s = *Standard solution* impurity peak response

C_s = Standard solution concentration of nutrient (mg/mL)

C_u = Sample solution concentration of nutrient (mg/mL)

F = relative response factor for the corresponding impurity peak

M_{r1} = L-5-methyltetrahydrofolic acid molecular weight

M_{r2} = **Calcium L-5-methyltetrahydrofolate molecular weight**

The relative retention times, relative response factors and acceptance criteria are given in **Table 2**.

Table 2: Relative retention times, relative response factors and acceptance criteria for impurities

Name	Relative Retention Time	Relative Response Factor	Acceptance Criteria, NMT (%)
4-Aminobenzoylglutamic acid	0.29	0.91	0.5
4a-Hydroxy-5-methyl tetrahydrofolic acid	0.37	1.09	1.0
(6R)-Mefox	0.49	1.05	-
(6S)-Mefox	0.50	1.05	1.0 (sum of 6R and 6S)
Tetrahydrofolic acid	0.65	1.00	0.5
7,8-Dihydrofolic acid	0.83	0.95	0.5
Folic acid	0.85	0.83	0.5
5,10-Methylenetetrahydrofolic acid	0.88	1.00	0.5
5-Methyltetrahydrop teroic acid	1.10	0.67	0.5
Dimethyltetrahydrofolic acid	1.25	1.00	0.15
Total impurities	-	-	2.5

Free Folate Levels

Free Folate levels refer to the concentration of folate present in a nutrient in a form that is readily available for absorption and utilization by the body. This measurement is essential for assessing the potency and effectiveness of the folate supplement and ensuring that it provides an adequate amount to meet the dietary needs of the individual consuming it. The Free Folate levels were calculated as follows:

For L-5-MTHF Calcium Forms

Free folate= A1 * (1-W)* M1/M2

For Quatrefolic® L-5-MTHF Glucosamine

Free folate= A2 * (1-W)* M1/M3

A1= Assay of L-5-MTHF Ca (on dry basis)

A2= Assay of L-5-MTHF Glucosamine (on dry basis)

W= water content

M1= L-5-MTHF molecular weight 459.46g/mol

M2= L-5-MTHF Ca molecular weight 497.5g/mol

M3= L-5-MTHF Glucosamine molecular weight 817.8g/mol

RESULTS AND DISCUSSION

The past decade has seen a significant rise in L-5-MTHF supplementation for the management of neurological conditions such as Alzheimer's disease, depression, and other mental health disorders. L-5-MTHF supplementation may provide numerous health benefits for individuals with low serum folate levels and/or elevated blood homocysteine levels. Water-soluble folate is one of the most crucial nutrients for metabolism because it serves as an essential cofactor in enzymatic processes that control amino acid and nucleic acid metabolism. Since neither humans nor animals are capable of producing folate on their own, they must regularly consume folate through diet to maintain sufficient levels. Folate deficiency during early pregnancy is associated with congenital malformations including neural-tube defects and congenital heart defects **[13]**. (6S)-5-methyltetrahydrofolate is a natural alternative to folic acid that may offer significant health benefits **[14]**. (6S)-5-MTHF is particularly sensitive to oxygen and moisture, which reduces the stability of the nutrient. The screening of stable crystalline salts is imperative as the crystal form of the nutrient affects its pharmacological action, bioavailability, dissolution, stability, and shelf life **[15]**. Therefore, this study evaluated the differences in changes in stability and physical appearance, content assay, total impurities, water content, and Free Folate levels of various brands of L-MTHF as well as the amorphous form.

Evaluation of stability in terms of physical appearance is an important parameter to ensure the quality of raw materials **[13]**. When Sample 1 was exposed to room temperature and relative humidity (65%), the color changed slightly in the first 14 days and then changed from white to light-yellowish on day 15. The appearance remained the same through to day 90. For Sample 2, the color changed from bright yellow to deep yellow from day 11, then to dark brown after day 45. For Sample 3, the color changes were similar to Sample 1, while Sample 3 had a more uniform appearance and the presence of large chunks of material were not seen. The color of Sample 4 changed to deep yellow within a few days. The changes in physical appearance are shown in **Figure 1**.

The X-Ray Diffraction results revealed that the characteristic peaks observed for (6S)-5-MTHF calcium salt in sample 3 were identical to the peaks highlighted in the XRD of the patented form in sample 1. **Figure 2** shows a comparison of the XRD pattern of Sample 3 with the pattern of Patented Brand Sample 1 **[12]**. The strongest peak Theta 2 values are also shown below.

To determine the identity as well as potency of a nutrient, it is necessary to conduct a content assay of the raw material **[13]**. The results of the content assay demonstrated that Sample 2 and Sample 4 declined sharply over the days as compared to the other two samples. The content assay for Sample 2 decreased sharply by 7.2% through to day 90, whereas Sample 4 declined significantly by 23.2% through day 90. During the period of 0-90 days, there was a nonsignificant change in the content assay for Sample 1 and Sample 3. A detailed description is given in **Table 3** and HPLC spectrograms in **Figure 3**.

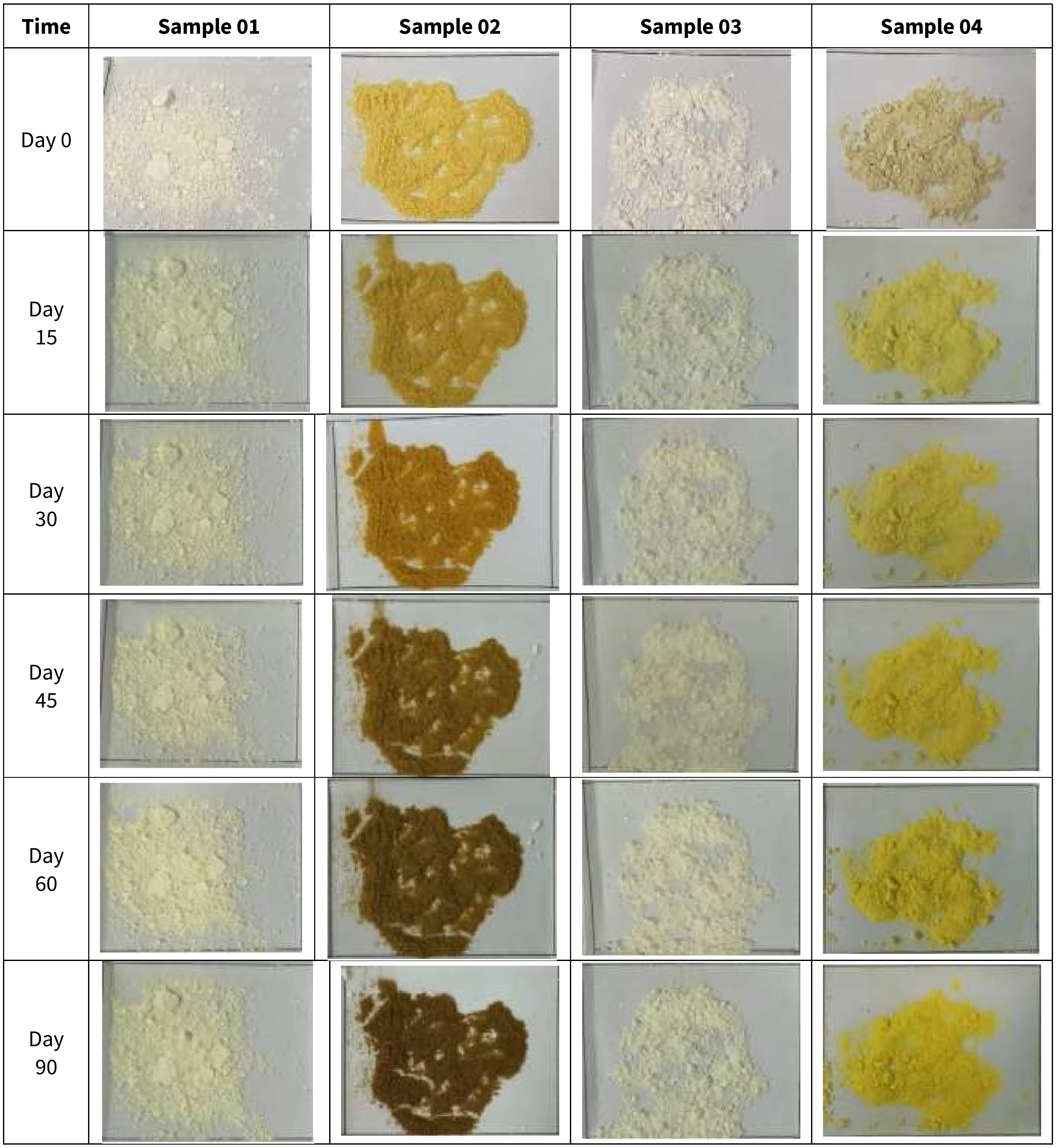
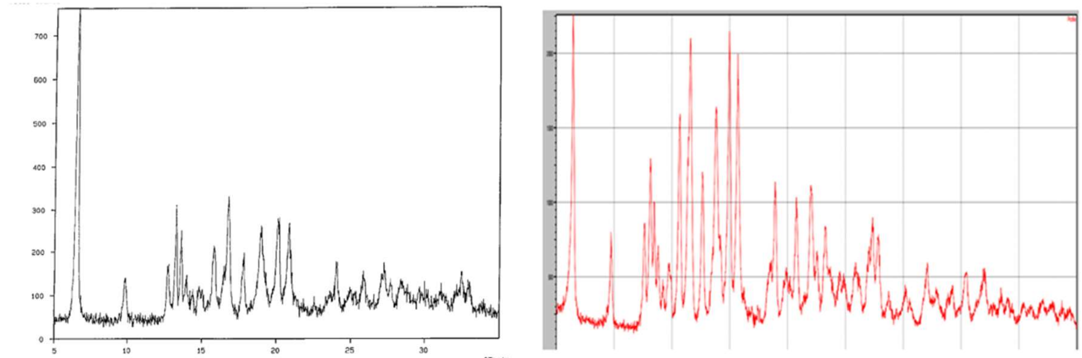


Figure 1: Changes in Physical Appearance of Samples on days 0, 15, 30, 45, 60, and 90



X Ray Diffraction Pattern for Patent Brand Metafolin (Sample 1)

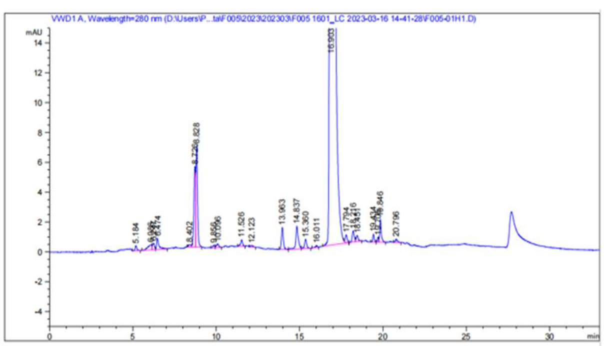
X Ray Diffraction Pattern for Cerebrofolate (Sample 3)

Samples	Characteristic Theta 2 Values
Sample 1	6.5, 13.3, 16.8, 20.1
Sample 3	6.44, 13.7, 16.5, 19.9

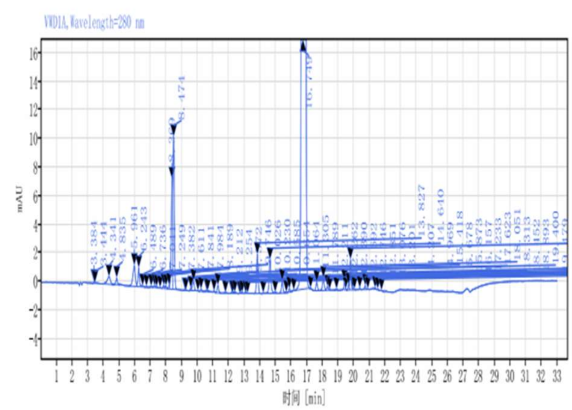
Figure 2: X-Ray Diffraction Patterns for Metafolin® and Cerebrofolate™

Table 3: Comparison of Content Assay on Days 0, 15, 30, 45, 60, and 90

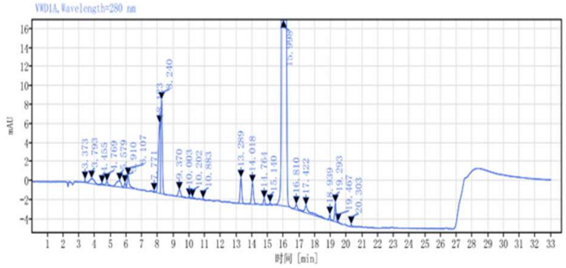
Samples	Day 0	Day 15	Day 30	Day 45	Day 60	Day 90
1	**98.0%**	**97.2%**	**98.1%**	**98.6%**	**98.5%**	**97.2%**
2	**57.2%**	**56.1%**	**57.5%**	**56.4%**	**54.9%**	**50.2%**
3	**98.5%**	**100.7%**	**99.9%**	**100.0%**	**100.2%**	**99.9%**
4	**79.9%**	**76.8%**	**70.2%**	**67.8%**	**63.8%**	**56.7%**



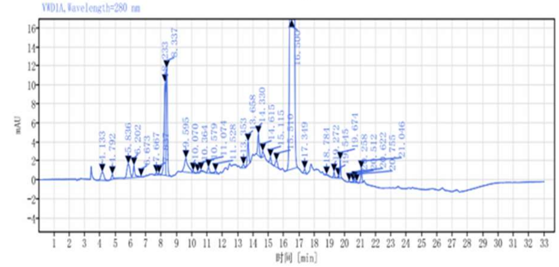
Day 0



Day 30

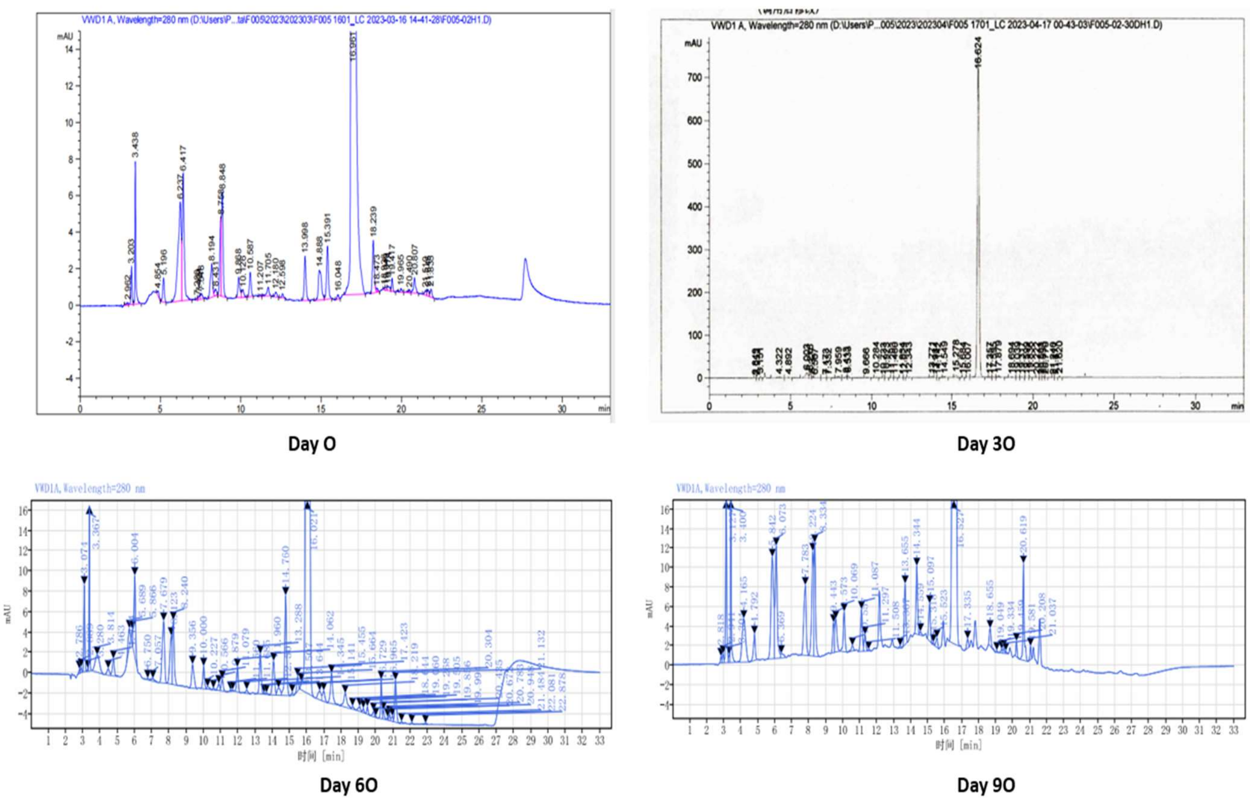


Day 60

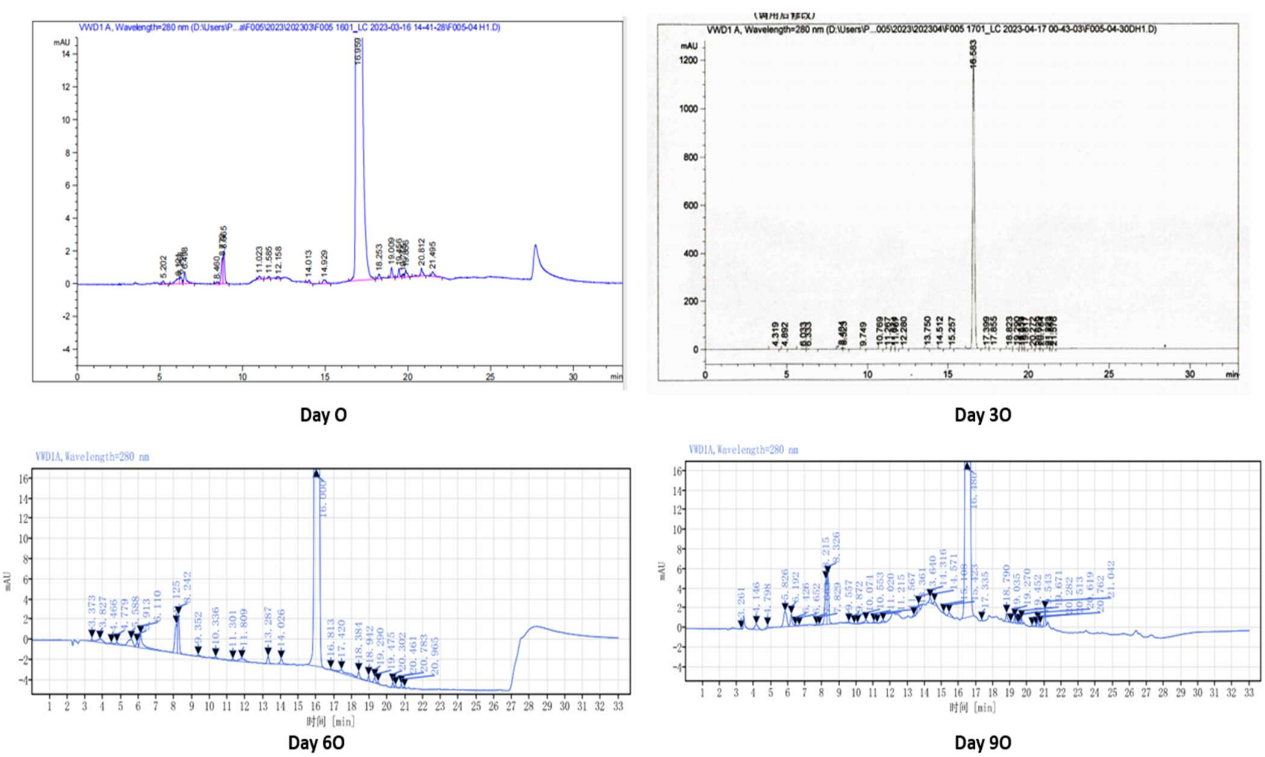


Day 90

Changes in HPLC Spectrograms for Sample 1



Changes in HPLC Spectrograms for Sample 2



Changes in HPLC Spectrograms for Sample 3

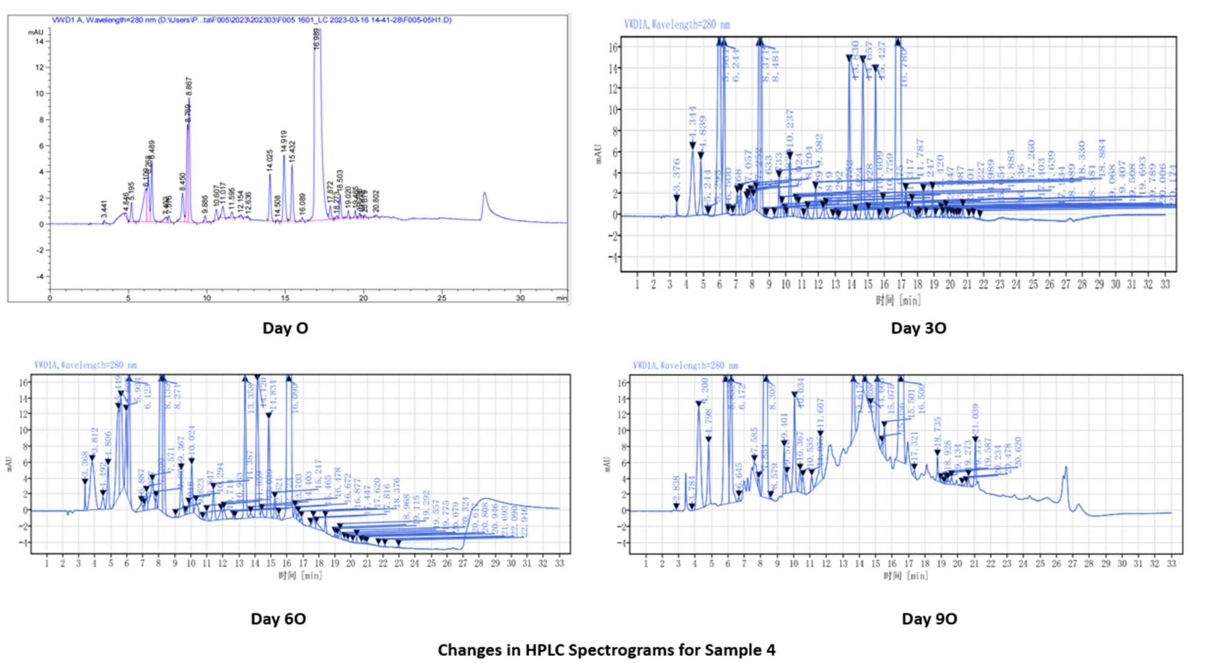


Figure 3: Comparison of HPLC Spectrograms on Day 0, Day 30, Day 60 and Day 90

It is crucial to identify and measure contaminants in pharmaceutical raw materials to ensure effectiveness and safety **[16]**. The results of the study showed that the total impurities content for samples 2 and 4 were comparatively higher on Day 0, 15, 30, 45, 60, and 90. The impurities content increased by 6.6% for Sample 2 through to day 90 whereas for Sample 4 the total impurities increased the most and showed the greatest change (18.5%) through to day 90. The increase in total impurities content was relatively nonsignificant for Sample 1 and Sample 3 as compared to the other samples. A detailed description is given in **Table 4 and Figure 4**.

The results of the study showed that the Free Folate levels (indicating nutrient potency) were highest for Sample 3 followed by Sample 1 on days 0, 15, 30, 45, 60, and 90. The Free Folate levels declined sharply for Sample 4 by 17.6% through to day 90, and moderately declined for Sample 2 (4.5%). Sample 3 ranked first whereas Sample 2 ranked the lowest(in terms of potency) on Day 90. A detailed description is given in **Table 5 and Figure 5**.

Table 4: Comparison of Total Impurities on Days 0, 15, 30, 45, 60, and 90

Samples	Day 0	Day 15	Day 30	Day 45	Day 60	Day 90	% of increase in Total Impurities from Days 0-90
1	1.8%	2.1%	2.1%	2.2%	2.3%	2.6%	0.8%
2	7.0%	9.0%	8.4%	9.4%	10.4%	13.6%	6.6%
3	0.68%	1.1%	1.2%	1.4%	1.3%	1.5%	0.8%
4	5.0%	12.1%	17.2%	20.2%	22.3%	25.3%	20.3%

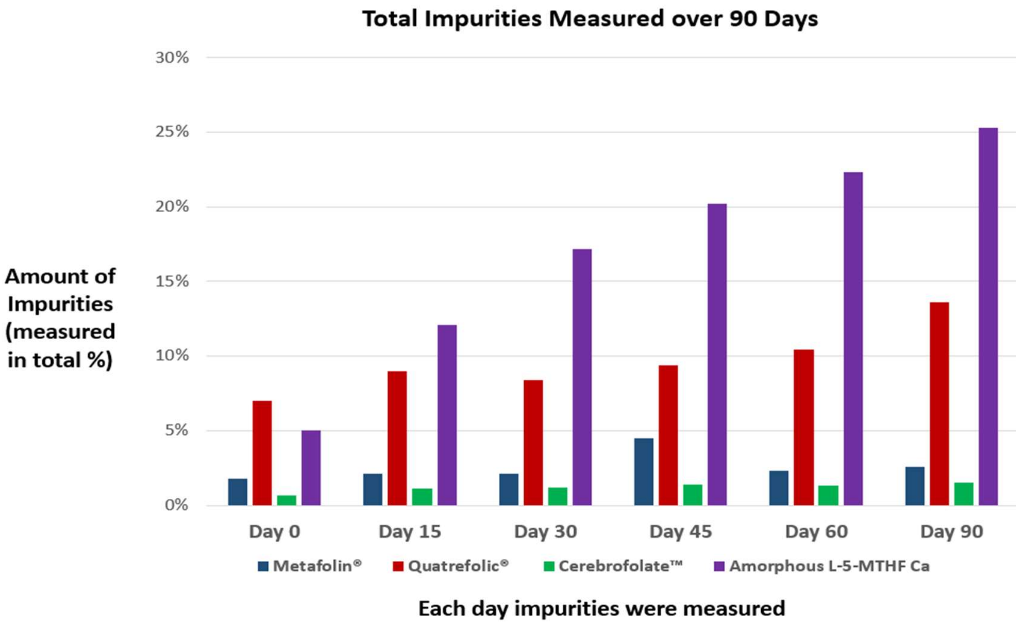


Figure 4: Comparison of Total Impurities on Day 0, 15, 30, 45, 60 and 90

Table 5: Comparison of Free Folate Levels indicating Potency on Days 0, 15, 30, 45, 60 and 90

Samples	Day 0	Day 15	Day 30	Day 45	Day 60	Day 90	Folate Potency Levels Ranked (Highest to Lowest)
1	78.3%	76.2%	76.7%	76.8%	77.3%	76.8%	NO.2
2	46.6%	46.0%	47.0%	45.7%	44.9%	42.1%	NO.4
3	80.3%	79.2%	78.7%	78.8%	78.9%	78.5%	NO.1
4	61.4%	55.9%	51.3%	49.4%	46.5%	43.8%	NO.3

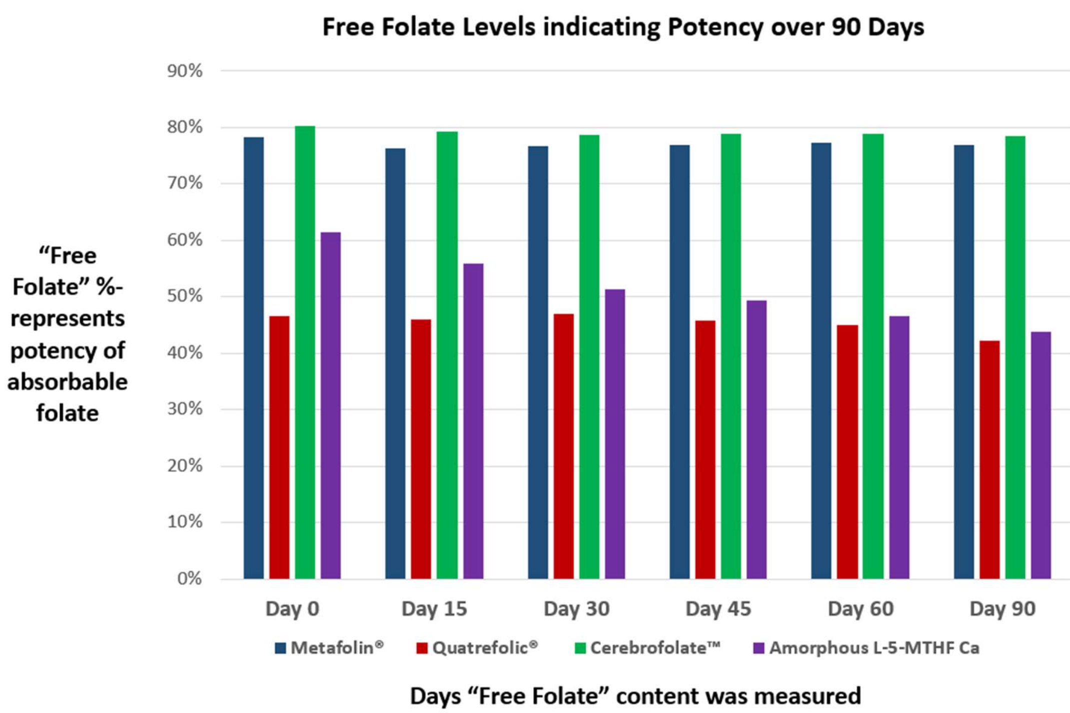


Figure 5: Comparison of Free Folate Levels indicating Potency on Days 0, 15, 30, 45, 60 and 90

CONCLUSION

The present study concluded that Cerebrofolate™ and Metafolin® were chemically identical, more stable, and had higher potency as compared to both the amorphous form of L-5-MTHF Calcium salt and the patented glucosamine salt form. The 90-day testing period demonstrated minimal change in the physical appearance, content assay, total water content, and total impurities for both the Cerebrofolate™ and Metafolin® samples. Both brands' samples had higher Free Folate levels in comparison to the other samples, indicating greater potency. It is imperative that manufacturers of L-5-MTHF ensure their product meets all quality control criteria and remains therapeutically active over time.

Conflict of Interest

The authors have no conflict of interest.

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