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Original Article

PHARMACOKINETIC STUDY OF HY-FOLIC® AND FOLIC ACID IN HEALTHY VOLUNTEERS

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ABSTRACT

Objective: HY-FOLIC® is the active form of Folic Acid (FA) produced by PT Simex Pharmaceutical Indonesia containing (6*S)*-5-Methyltetrahydrofolate *(*5-MTHF). We evaluated the pharmacokinetic profiles of HY-FOLIC® versus FA after single oral administration in healthy volunteers.

Methods: A randomized, open-label, 2-way crossover, single-dose design was conducted on 12 healthy subjects with two steps. In the first step, 6 subjects were given 1100 mcg of HY-FOLIC®(1.3 µmol), and 6 subjects were given an equimolar dose of 600 mcg of FA (1.3 µmol) in a fasting condition. Blood samples were taken before, and at 1, 2, 3, 4, 5, 6, 7, and 8 h after administration of products for measurement of peak concentration (Cmax), the Area Under the Curve at t-time (AUCt) and infinite time (AUCinf). After a washout period of 14 d, the same procedure was repeated in which the first 6 subjects received FA, and the second 6 subjects received HY-FOLIC®. Pharmacokinetic data of 5-MTHF and *Unmetabolized Folic Acid* (UMFA) were compared with paired t-tests.

Results: Cmax of 5-MTHF (mean±SD) following administration of HY-FOLIC®and FA were 46.91+28.16 vs 22.61+15.73 nmol/l, respectively (p=0.000); the AUCt were 214.47+183.46 vs 112.93+112.11 h. mmol/l (p=0.001). Conversely, Cmax of UMFA were 9.49+7.89 vs 21.97+14.79 nmol/l (p=0.003); AUCt 33.29+39.34 vs 78.16+58.93 h. nmol/l (p=0.001).

Conclusion: HY-FOLIC®is much more bioavailable than FA as indicated by much higher Cmax and AUCt of the active form of 5-MTHF.

Keywords: Folic acid, HY-FOLIC®, Methyltetrahydrofolate, Pharmacokinetics

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INTRODUCTION

Folic Acid (FA) or vitamin B9, is a water-soluble vitamin that plays a significant role in *Deoxyribonucleic Acid* (DNA) synthesis and methylation reactions. This vitamin is found in vegetables, food, and supplements. FA is used for red blood cell production and maintaining normal cellular growth and function. FA is not synthesized within the body; its provision is derived from food or external supplements. Consumption of FA in pregnancy is essential to prevent brain and spine defects such as spina bifida [1, 2] as well as to prevent anemia in the mother when combined with iron [3]. In addition, the Indonesian government has also implemented mandatory folate fortification for pregnant women. Methotrexate treatment for various conditions such as rheumatoid arthritis and malignancy also needs FA or folinic acid supplementations to reduce adverse drug reactions [4-6].

In the human body, FA must be converted to its biologically active form, 5-Methyltetrahydrofolate (5-MTHF), the major form of folate in blood circulation. Before entering the folate cycle, FA is converted to *dihydrofolate* by Dihydrofolate Reductase (DHFR), and then further reduced to *tetrahydrofolate*. Tetrahydrofolate is subsequently converted to 5-MTHF, the active form of folate, by Methyltetrahydrofolate Reductase (MTHFR) [4, 5].

Consumption of FA is associated with different concentrations of its active metabolite due to polymorphism characteristic of DHFR activity [9, 10], leading to different efficacy in preventing MTX side effects. Supplementation of folate may be obtained by consumption of either FA, folinic acid, or 5-MTHF, the direct active form of FA. Bailey and Ayling (2018) reported that the administration of 5-MTHF was associated with more rapid attainment of total folate therapeutic concentration compared to the administration of FA in pregnant women [11]. Thus, the use of 5-MTHF is strongly recommended over FA for external supplementation [12]. Obeid *et al.* [13] reported that Cmax and AUC of 5-MTHF were higher after consumption of an equimolar dose of calcium salt of (6S)-5-MTHF acid compared to consumption of FA.

HY-FOLIC® is a film-coated tablet formulated as glucosamine salt of (6S)-5-MTHF, produced by PT Simex Pharmaceutical Indonesia. It is available as an 1100 mcg film-coated tablet which is equimolar to 600 mcg of folic acid. The present study aims to evaluate the pharmacokinetic profile of a single dose of HY-FOLIC®in comparison with an equimolar dose of FA.

MATERIALS AND METHODS

Study products

Both HY-FOLIC® and FA were supplied by PT. Simex Pharmaceutical Indonesia. The two compounds were provided in identical capsules and packaged for individual subjects according to a pre-determined randomization list. Each package was labeled with sequential subject numbers and dosage instructions.

Study design

This was a randomized, open-label, 2-way crossover, single-dose study with a 14 d wash-out period in 12 healthy subjects under fasted conditions, conducted at the Pharma Metric Laboratory, Indonesia, between February and March 2023. The methods of the study followed the 2010 European Medical Agency (EMA) guideline of Bioequivalence study [14].

The study test products consisted of 1100 mcg (1.3 µmol) (6S)-5-MTHF glucosamine salt (HY-FOLIC®), and 600 mcg FA (1.3 µmol). Both folate compounds were provided in odorless gelatin capsules of identical appearances.

Ethics of the study

The protocol of this study has been approved by the Health Research Ethics Committee at the Faculty of Medicine, Universitas Indonesia with letter number: KET-58/UN2. F1/Etik/PPM.00.02/2023.

The study was done in compliance with the Declaration of Helsinki (World Medica Association), 2000 and Indonesian Guideline of Good Clinical Practice. Written informed consent was obtained from each subject before participating in the study.

Inclusion criteria

The subjects of this study were healthy males and females aged 18 to 35 y, with normal blood pressure (systolic 90-120 mmHg and diastolic 60-80 mmHg), a heart rate of 60-100 bpm, a BMI of 18.5-25 kg/m^2, and normal plasma folate levels (7-45 nmol/l).

Exclusion criteria

Pregnant or breastfeeding women, as well as those intending to become pregnant during the study, were excluded. Additionally, individuals with diseases that could potentially interfere with folate absorption or metabolism (such as gastrointestinal disorders, diabetes, renal insufficiency, hepatic dysfunction, cardiovascular disease, or cancer) were excluded. Other exclusion criteria included the presence or history of any coagulation disorders, clinically significant hematologic abnormalities (e. g., anemia with hemoglobin levels<12.0 g/dl in women and<13.0 g/dl in men), malnutrition (BMI<18.5 kg/m^2 or>25 kg/m^2), smoking more than 10 cigarettes per day, participation in any clinical study within the past 3 mo, or donation or loss of 300 ml or more of blood within the 3 mo preceding the study.

Further exclusions included individuals who tested positive for the SARS-CoV-2 antigen or had direct contact with a COVID-19-positive person in their neighborhood within 3 days before screening. Those with a history or current symptoms of sore throat, fever (temperature>37 ℃), or dyspnea within the last 14 d, as well as those with a history of drug or alcohol abuse within the 12 mo before screening, were also excluded. Consumption of Mono Sodium Glutamate (MSG) or its derivatives within 24 h before participation, as well as supplements containing folic acid in the last 3 mo or drugs or compounds that could interfere with folate status or assay (such as proton pump inhibitors, histamine-2 receptor blockers, antacids, anticoagulants, methotrexate, trimethoprim-sulfamethoxazole, antidepressants, and antiepileptic drugs) within the last 2 w, was not allowed.

Study subjects

The subjects were healthy volunteers who routinely participated in bioequivalence studies at the Pharmametric lab. Simple random sampling was used to allocate subjects to receive either HY-FOLIC® or FA during the first period. As this study employed a crossover design, all subjects eventually received both products, with each subject serving as their own control.

Sample size determination

We used 12 subjects with crossover design to measure the Cmax, Tmax, and AUCt of 5-MTHF following the administration of HY-FOLIC® versus FA. According to the EMAGuideline of Bioequivalence study, the minimum number of evaluable subjects should not be less than 12 subjects [14]. Thus, in this study, we conducted a preliminary analysis with 12 subjects. The adequacy of the sample size was then verified by applying the results to the formula for sample size calculation in paired comparisons between two groups: $n1 = n2 = \{(Za+Zb)\ S\}^2/\{(X1-X2)\}^2$ [15].

Study procedure

Written informed consent was obtained from each subject before screening. Data collected from candidates included demographic information, medical history, concomitant medications, and COVID-19 vaccination status. After a physical examination, a 15 ml blood sample was taken for routine hematology, plasma folate concentration, and tests for HIV, HBsAg, and HCV.

Pretreatment period

The subjects were instructed to abstain from taking any medication for at least one week before and during the study period unless it was medically necessary. For 24 h before the study day and throughout the entire sampling day, the subjects were not permitted to smoke or consume alcohol, milk, beverages, or foods containing xanthine, such as tea, coffee, chocolate, cola, or fruit juice. Foods containing MSG and its derivatives (*disodium 5′-ribonucleotides, disodium inosinate*, and *disodium guanylate*), such as soy sauce, ketchup, chili sauce, processed meat, or mushrooms, were also not allowed.

Food intake during the study period was standardized for all subjects. On the previous evening, the subjects were confined to the study site. They were not allowed to bring any food or drink to be consumed at the study site. Water was allowed as desired, except for 1 hour before until 2 h after drug administration.

Treatment period

Following a 12 h fast, the subjects underwent a physical examination to collect baseline vital sign data, including body temperature, blood pressure, heart rate, and respiration rate. Female subjects of child-bearing age were required to undergo a urinary pregnancy test.

After the examination, the subjects consumed the study products with 150 ml of water: 6 subjects consumed one tablet of the test drug (HY-FOLIC®), and the other 6 subjects consumed one tablet of FA as the reference drug. They were instructed to remain in an upright position (standing or sitting) for one hour after product intake.

An amount of 6 ml of blood was withdrawn via venipuncture using a disposable sterile syringe before drug administration (0 h) and at 1, 2, 3, 4, 5, 6, 7, and 8 h after product intake. In total, 54 ml of blood was withdrawn from t_0 to t_8, and all the blood samples were placed in tubes containing K^+-EDTA. After a 14 d wash-out period, the same procedures were repeated, with the first 6 subjects receiving one tablet of FA and the second 6 subjects receiving one tablet of HY-FOLIC®. The collected blood samples were centrifuged at 3000 rpm for 10 min using a centrifuge with a 32 cm rotor diameter. The plasma was then separated and stored frozen at a maximum temperature of-20 ℃ until assayed.

Bioanalytical and statistical analysis

Plasma concentrations of 5-MTHF and UMFA were measured using a validated LC-MS/MS method. The calculation of drug level was performed using a 5-MTHF and UMFA calibration curve based on the peak ratios of the analyte versus the internal standard used at the specified concentrations.

The pharmacokinetic parameters of 5-MTHF and UMFA measured were Cmax (maximum concentration), Tmax (time required to reach the maximum concentration), AUCt (area under the concentration-time curve from time 0 to 8 h), AUCinf (area under the concentration-time curve from time zero to infinity), and T½ (the elimination half-life).

Pooled data of 5-MTHF and UMFA after consumption of HY-FOLIC® or FA were compared using a paired t-test following the logarithmic transformation of the main parameters (AUCt and Cmax).

RESULTS

Demographic characteristics

There were 12 subjects (2 females and 10 males) completed this pharmacokinetic study, with a mean age of 28 y (ranging from 24 to 31 y). Mean weight was 61 kg (53 to 71 kg), mean height was 168 cm (160 to 172 cm), and mean BMI was 21.41 kg/m^2(18.56 to 24.86 kg/m^2). The complete demographic data can be seen in table 1.

Pharmacokinetics of 5-MTHF

The mean±SD of Cmax of 5-MTHF after HY-FOLIC® and FA intake was 46.91±28.16 nmol/l and 22.61±15.73 nmol/l, respectively (p = 0.000). The Cmax ratio of 5-MTHF between HY-FOLIC® and FA consumption was 207.49%. The AUCt values were 214.47±183.49 nmol·h/l and 112.93±112.11 nmol·h/l, respectively (p = 0.001), and the ratio of AUCt between the two groups was 189.92% table 2 and fig. 1.

Table 1: Demographic data of the study subjects

Demographic data	n (%)	Mean±SD
Male	10 (83.33)	
Female	2 (16.67)	
Age (y)	-	28±2.48
Weight (kg)	-	61±6.22
BMI (kg/m2)	-	21.41±2.73
Smoker	5 (41.67)	
Non-smoker	7 (58.33)	
Systolic BP	-	114±6.11
Diastolic BP	-	78±4.02
Heart Rate	-	81±16.56

Data are presented as n (%) and mean±SD when appropriate, n = 12

Table 2: Pharmacokinetic data of 5-MTHF after consumption of HY-FOLIC® vs folic acid

Pharmacokinetic parameters of 5-MTHF	Test drug HY-FOLIC®	Reference drug Folic acid	P values*
C_{max} (nmol/l)	46.91±28.16	22.61±15.73	0.000
AUCt (h. nmol/l)	214.47±183.49	112.93±112.11	0.001
AUCinf (h. nmol/l)	403.26±427.61	228.99±260.77	0.059
T_{max} (h)	2.33±1.37	2.83±1.64	0.520
$T_{½}$ (h)	5.78±6.98	4.89±6.04	0.755

*Paired t-tests. Data are presented as mean±SD, n =12

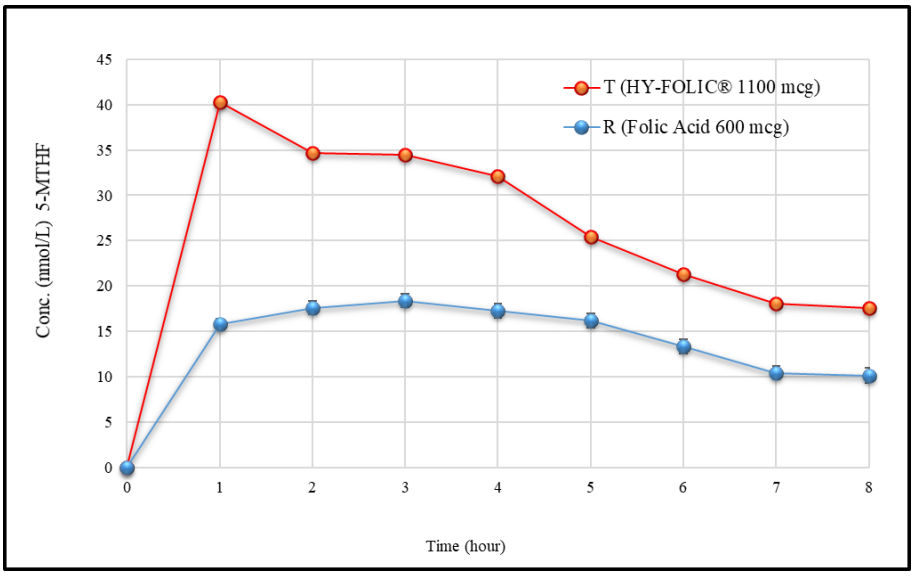

Fig. 1: Mean plasma concentrations vs time of 5-MTHF in the group receiving HY-FOLIC® and Folic Acid

Kinetics of unmetabolized folic acid (UMFA)

Table 3 shows the pharmacokinetic data of UMFA after intake of HY-FOLIC® and FA, and fig. 2 illustrates the mean peak plasma concentration. UMFA was detected in all 12 subjects after FA intake, but in only 9 out of 12 subjects after HY-FOLIC® intake. We observed that the Cmax, AUCt, and AUCinf of UMFA were significantly lower after the intake of HY-FOLIC® compared to FA (table 3 and fig. 2. Tmax of UMFA was longer after the administration of HY-FOLIC® compared to FA, although the difference was not statistically significant.

Table 3: Pharmacokinetic data of UMFA after consumption of HY-FOLIC® vs folic acid

Pharmacokinetic parameters of UMFA	Test drug HY-FOLIC®	Reference drug folic acid	P values*
C_{max} (nmol/l)	9.49±7.89	21.97±14.79	0.003
AUC_t (h. nmol/l)	33.29±39.34	78.16±58.93	0.001
AUC_{inf} (h. nmol/l)	52.22±62.42	125.39±111.15	0.002
T_{max} (h)	3.0±2.71	1.67±0.65	0.101
$T_{½}$ (h)	2.42±2.37	4.24±5.31	0.281

Data are presented as mean±SD, n = 12

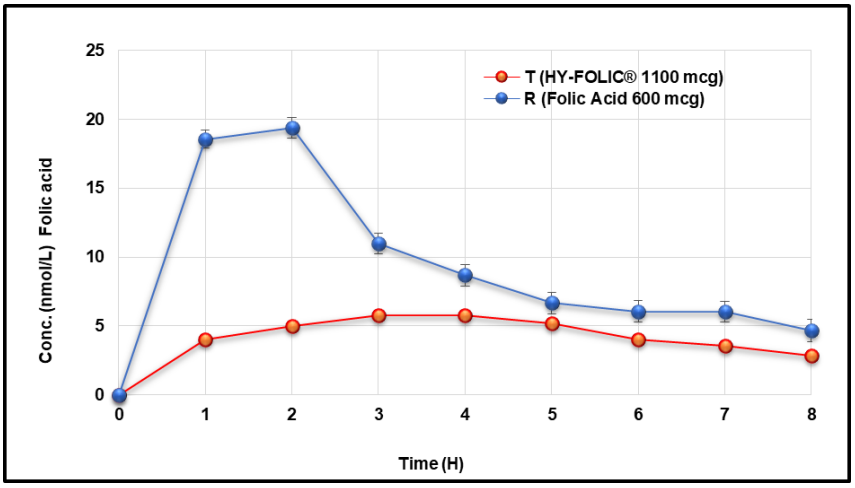

Fig. 2: Mean plasma concentrations vs time of Folic Acid in the group receiving HY-FOLIC® and Folic Acid (n = 12),

data are presented as mean, n = 12, the mean±SD of the T½ of 5-MTHF was slightly higher after the administration of HY-FOLIC® (5.78±6.98 h) compared to FA (4.89±6.04 h), although this difference was not statistically significant. The mean±SD values of T½ for UMFA were 2.42±2.37 h after HY-FOLIC® and 4.24±5.31 h after FA

DISCUSSION

In the present study, we report the results of a pharmacokinetic study of 5-MTHF, the biologically active metabolite of FA, following the oral administration of 600 mcg of FA or an equimolar dose of HY-FOLIC®. HY-FOLIC® contains 1100 mcg (1.3 µmol) of (6S)-5-MTHF acid as a glucosamine salt. It was revealed that Cmax and AUC of 5-MTHF were significantly higher after administration of HY-FOLIC®compared to FA consumption.

In the human body, FA needs to be converted to its biologically active form. Before entering the folate cycle, FA undergoes hepatic metabolism through several steps. First, FA is converted to *dihydrofolate* and then to *tetrahydrofolate* by DHFR. Next, *tetrahydrofolate* is converted by MTHFR to 5-MTHF, which is the major active form of folate in blood circulation [7, 8].

FA is commonly used in several conditions such as pregnant women which has been proven beneficial to prevent neural tube defects [1, 2, 4]. Another common use of FA is in patients who receive high doses of methotrexate, such as rheumatoid arthritis and cancer [5, 6], to prevent folate deficiency. In addition, folate supplementation has been shown to reduce the risk of cardiovascular disease through its effect of reducing hyperhomocysteinemia [3].

The rate of conversion of FA into *dihydrofolate* and *tetrahydrofolate* is not uniform in all individuals because the DHFR enzyme exhibits polymorphism characteristics. Individuals with low DHFR activity, such as those with the 19-bp mutation, show a lower capacity to activate Folic Acid [17]. This type of mutation has been associated with preterm neural defects, such as spina bifida [17]. In patients undergoing methotrexate treatment, polymorphism of the DHFR gene may affect treatment outcomes and the occurrence of adverse effects of methotrexate [17, 18]. Thus, the administration of 5-MTHF as an active metabolite, instead of the parent drug of Folic Acid, can be considered a practical solution for addressing this polymorphism problem.

The mean Cmax and AUCt of 5-MTHF in subjects who received HY-FOLIC® were higher than those who received FA, with a ratio of 207.49% and 189.92%, respectively, between the two products (table 2 and fig. 1). This signifies that the consumption of the active product (HY-FOLIC®) is much more efficient compared to the consumption of the parent drug Folic Acid.

Plasma levels of 5-MTHF were detected in all subjects after the administration of HY-FOLIC®, whereas it was not detected in one subject who received FA. It is unclear whether this anomaly has a genetic background associated with low or even zero activity of dihydrofolate reductase. Non-consumption of the product by this particular subject can be ruled out, as the drug intake by all subjects was closely observed by two laboratory assistants. Malabsorption remains another possibility.

Conversely, plasma concentrations of UMFA were detected in all 12 subjects after intake of FA, while it was only detected in 9 subjects after intake of HY-FOLIC®. The Cmax and AUCt of UMFA were significantly lower following intake of HY-FOLIC® compared to FA. The origin of UMFA after intake of HY-FOLIC® is not well known, as the normal conversion occurs from FA to 5-MTHF [7, 8], but not the reverse. The possibility of alternative pathways remains to be elucidated.

The mean T½ value of 5-MTHF was 5.78±6.98 h after administration of HY-FOLIC®, which was slightly, but not significantly, higher than that after administration of FA (4.89±6.04 h). Conversely, the mean T½ value of UMFA was lower after the administration of HY-FOLIC® (2.42±2.37 h) compared to FA (4.24±5.31 h), although this difference was not statistically significant.

Another issue concerning the consumption of HY-FOLIC® is its acceptability and potential side effects. During this study with single-dose administration, no adverse events were detected. Further studies with repetitive doses are warranted to assess the long-term safety profile.

The main limitation of this study was the small sample size which included only 12 subjects. However, the adequacy of this sample size was verified by recalculating the Cmax of 5-MTHF using the sample size formula, comparing the administration of HY-FOLIC® to FA. It was revealed that at least 9 subjects were needed per group to achieve 90% power ($\alpha = 0.05$). Thus, the use of 12 subjects in the present study is considered sufficient and is in line with the 2020 EMA guideline, which necessitates not fewer than 12 subjects [14]. The crossover design enabled each subject to serve as his/her own control, and comparison using the paired t-test showed a highly significant difference in Cmax and AUC of 5-MTHF after administration of HYFOLIC® versus FA.

CONCLUSION

The results of this study showed that the Cmax and AUCt of 5-MTHF were higher after a single oral dose of HY-FOLIC® compared to the equimolar dose of FA. It can be concluded that the consumption of HY-FOLIC® is much more bioavailable than Folic Acid.

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AUTHORS CONTRIBUTIONS

NN performed the drafting of the article, made interpretation of data, contributed to intellectual content, and made final approval. FDS conceived the design of the study, performed analysis and interpretation of data, made critical revisions of the manuscript, and final approval of the article.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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