Calculation of red blood cell folate steady state conditions and elimination kinetics after daily supplementation with various folate forms and doses in women of childbearing age$^{1,2}$

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ABSTRACT
Background: A maternal red blood cell (RBC) folate concentration $> 906\, \text{nmol/L}$ is thought to be optimal for lowering the risk of neural tube defects (NTDs) in pregnancy. Whereas the appearance of folate in RBCs has been followed during folic acid supplementation, data on elimination kinetics are not yet available.

Objective: The aim of our investigation was to estimate the steady state conditions and elimination kinetics of folate in RBCs.

Design: Data from 2 randomized, placebo-controlled, double-blind intervention trials were used for kinetic modeling. These studies were performed to investigate the appearance of folate in RBCs in healthy women of childbearing age after different supplementation strategies (study 1: $n = 69$; $400\, \mu\text{g}$ folic acid/d and $416\, \mu\text{g}$ [6S]-5-methyltetrahydrofolate/d for 24 wk; study 2: $n = 21$; $800\, \mu\text{g}$ folic acid/d for 16 wk).

Results: For RBC folate concentrations, steady state conditions were not reached after 24 (study 1) and 16 (study 2) wk of folate supplementation. However, with the use of these data, we calculated the biological half-life ($t_{1/2}$) of RBC folate to be $\approx 8$ wk. With the application of pharmacokinetic principles, steady state conditions for RBC folate should be reached after $40$ wk ($t_{1/2} \times 5$).

Conclusion: With the use of pharmacokinetic principles, the appearance and elimination kinetics of RBC folate can be calculated on the basis of this $t_{1/2}$ value.

KEY WORDS Red blood cell folate, steady state, elimination, kinetics, neural tube defects

INTRODUCTION

Low maternal folate status is related to a greater risk of adverse pregnancy outcomes, such as neural tube defects (NTDs) and early spontaneous abortion (1–3). Supplementation with folic acid before conception and during the first trimester has been shown to reduce the risk of NTDs and other adverse pregnancy outcomes by up to 100% (4–7). Health authorities recommend that women of childbearing age take a daily supplement containing $400\, \mu\text{g}$ folic acid/d for primary NTD prevention (8, 9) and $800\, \mu\text{g}$ folic acid/d for secondary prevention of NTDs (8, 9). In Europe, the incidence of NTDs remained $\approx 4500$ cases/y during the past decade (10).

Using red blood cell (RBC) folate as a marker of long-term folate status, women with an RBC folate concentration $> 906\, \text{nmol/L}$ were found to have the lowest risk of an NTD-affected pregnancy (1). The appearance of folate in RBCs during folate supplementation has been investigated in several intervention trials (11–14). However, none of these studies were of sufficient duration to achieve steady state RBC folate concentrations, nor did these studies evaluate elimination kinetics. According to pharmacokinetic principles, steady state conditions are reached after 5 half-life ($t_{1/2}$) periods. RBCs incorporate folate only during erythropoiesis, and they release the vitamin during their cell lyses. RBC folate is therefore thought to have a turnover similar to the lifespan of the RBCs, ie, $\approx 120$ d (15, 16). The aim of our investigation was to estimate the steady state conditions and elimination kinetics of folate in RBCs in healthy women after daily supplementation with various forms and doses of folate. The folate doses provided were $400\, \mu\text{g}$ folic acid/d (12) [this dose is recommended for primary NTD prevention (8, 9)] and an equimolar amount ($416\, \mu\text{g}$/d) of [6S]-5-methyltetrahydrofolate ([6S]-5-MTHF) and $800\, \mu\text{g}$/d folic acid (13) [this dose was shown in one study to reduce the NTD rate by 100% (5)].

SUBJECTS AND METHODS

Subjects and intervention

Data from 2 of our randomized, placebo-controlled, double-blind intervention trials (12, 13, 17) were used to calculate RBC folate kinetics. In both study 1 and study 2, the effect of daily supplementation with folic acid on RBC folate concentrations was investigated in healthy women of childbearing age. In study 1, a 24-wk intervention trial, we compared the efficacy of supplementation with $400\, \mu\text{g}$ folic acid/d and an equimolar amount ($416\, \mu\text{g}$/d) of [6S]-5-MTHF (12, 17). In study 2, subjects consumed a multivitamin supplement containing $800\, \mu\text{g}$ folic acid/d for 16 wk (13). Study 1 was supported by Merck KGaA (Darmstadt, Germany) and Merck Eprova AG (Schaffhausen, Switzerland); study 2 was supported by Roche Vitamins (Basel, Switzerland).

Inclusion criteria for participation in both studies were the following: age between 18 and 35 y, normal results on hematology and blood chemistry tests, and an adequate vitamin B-12 status (ie, a plasma vitamin B-12 concentration $> 110\, \text{ng/mL}$).

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pmol/L). Exclusion criteria were pregnancy, lactation, or planning of a pregnancy within the next few months; regular consumption of vitamin supplements containing folic acid or of food fortified with folic acid (>100 μg folic acid/d during the past 4 mo); recent medical treatment; and abuse of alcohol or drugs.

All subjects gave written informed consent. The Ethics Committee of the Medical Association of Hamburg (Hamburg, Germany) approved both studies.

Supplements

Supplements were in the form of capsules; one capsule was taken every morning before breakfast except on the blood sampling days, when the capsule was taken after venipuncture. The capsules were provided in numbered containers at baseline and at weeks 8 and 16 in study 1 and at baseline and at weeks 4, 8, and 12 in study 2. Subjects were randomly assigned to an intervention regimen according to the order of their arrival for blood sampling at baseline. Both subjects and investigators were blinded to treatment. Placebo groups were included for both studies.

For study 1, the supplements were manufactured by PCI Services (Schorndorf, Germany) as hard gelatin capsules, each containing a blend of magnesium stearate and microcrystalline cellulose as a filler ( placebo) and either 400 μg (906 nmol) folic acid (Caesar & Loretz GmbH, Hilden, Germany) or 416 μg (906 nmol) [6S]-5-MTHF as calcium salt (Metafolin; Merck Eprova AG, Schaffhausen, Switzerland). For study 2, capsules containing 800 μg (1812 nmol) folic acid were provided as multivitamin-multimineral preparation (Elevit Pronatal; Roche Consumer Health, Basel Switzerland; produced by Laboratories Roche-Nicholas, ITC Galenique, Gaillard, France). The capsules were film-coated and also contained vitamin A, thiamine, riboflavin, niacin, pantothenic acid, vitamin B-6, biotin, vitamin B-12, vitamin C, vitamin D, vitamin E, calcium, copper, iodine, iron, magnesium, and manganese. The filler for both multivitamin and placebo capsules was made up of microcrystalline cellulose, t alc, and magnesium stearate.

Biochemical measurements

Fasting blood samples were collected by venipuncture at baseline and at weeks 4, 8, 12, 16, 20, and 24 in study 1 and at baseline and at weeks 4, 8, 12, and 16 in study 2. For the measurement of RBC and plasma folate concentrations, fasting blood samples were collected into heparinized tubes. After measurement of the hematocrit, whole-blood samples for RBC folate analysis were centrifuged (2000 g) at 20 °C and stored in the dark before storage at 80 °C. Folate concentrations were measured by a microbiological assay (18). The intraassay and interassay CVs were 0.7% and 7.0% for whole-blood folate and 0.3% and 6.2% for plasma folate, respectively. For external validation, a whole-blood folate standard (National Institute for Biological Standards and Control, Potters Bar, United Kingdom) was analyzed at each run. To avoid between-assay variation, samples from each participant were measured in a single assay. RBC folate concentrations were calculated according to the following equation:

\[
\text{RBC folate} = \left[\text{whole blood folate} \times 100\right] - \left[\text{plasma folate} \times [100 - \text{hematocrit}()]/\text{hematocrit} (J)\right]
\]

For confirmation of general health, standard blood tests were performed at baseline and at the end of the intervention (week 24 and week 16 in study 1 and 2, respectively) and were analyzed by the central laboratory of the University Hospital, Bonn, Germany.

Dietary intakes were assessed by 3-d diet records completed at baseline and weeks 8, 16, and 24 in study 1 and at baseline and weeks 8 and 16 in study 2. They were analyzed by using EBISP RO for WINDOWS software (version 4; Jürgen Erhardt, University of Hohenheim, Stuttgart, Germany). Compliance with respect to the supplement intake was assessed by pill counting at weeks 8, 16, and 24 in study 1 and at weeks 4, 8, 12, and 16 in study 2.

Statistical analysis

The kinetic model is presented as part of the Results section. For comparison of the observed RBC folate concentrations with the estimated values derived from the kinetic model, arithmetic means and SDs were calculated. All analyses were undertaken by using SPSS for WINDOWS software (version 12; SPSS Inc, Chicago, IL).

RESULTS

Increase in RBC folate after folic acid or folate supplementation

In studies 1 and 2, the increase in RBC folate concentrations became progressively smaller (Figure 1) between each 8-wk intervention period, but did not appear to reach steady state. RBCs incorporate folate during erythropoesis. Thus, we expected the RBC folate to reach steady state after one lifespan of RBCs, ie, ≈120 d. The flattening effect of RBC folate increase observed at 8-wk intervals (56 d), however, it seems to adhere to the pharmacokinetic principle that steady state conditions are reached after 5 t1/2. Thus, the t1/2 of RBCs (60 d) seems to be identical to that of folate in RBCs.

![FIGURE 1. Mean (±SD) red blood cell (RBC) folate concentrations at baseline (□), week 8 (■), and week 16 (□), and week 24 (□) after daily supplementation with 400 μg folic acid/d (n = 34) or 416 μg [6S]-5-methyltetrahydrofolate ([6S]-5-MTHF)/d (n = 35) (study 1) and at baseline and weeks 8 and 16 with 800 μg folic acid/d (n = 21) (study 2).](https://academic.oup.com/ajcn/article-abstract/86/5/1414/4650671/FIGURE-1-Mean-+-SD-red-blood-cell-RBC-folate-concentrations-at-baseline-□-week-8-■-week-16-□-and-week-24-□-after-daily-supplementation-with-400-μg-folic-acid-d-n-34-or-416-μg-6S-5-methyltetrahydrofolate-6S-5-MTHF-d-n-35-study-1-and-at-baseline-and-weeks-8-and-16-with-800-μg-folic-acid-d-n-21-study-2)
Kinetic model

On the basis of pharmacokinetic principles, we hypothesize that RBC folate concentrations reach steady state conditions after 5 t_{1/2} of RBC lifespan after continuous folic acid or folate supplementation. In the second, third, fourth, and fifth t_{1/2}, RBC folate increases by one-half of the last total increase plus ΔC_{i1}; ie, ΔC_{ni} = ΔC_{(n-1)1/2} + ΔC_{i1} (Figure 2). A steady state (plateau) is reached after 5 t_{1/2}, as shown in Figure 3.

The appearance and steady state conditions were calculated according to the following equation:

\[ C(n) = C(0) + \mu \times (1 - 0.5^n)/(1 - 0.5) \]  

(2)

where \( C(n) \) is the RBC folate concentration after \( n \) t_{1/2}; \( C(0) \) is the RBC folate concentration at baseline, and \( \mu \) is the empiric increase in RBC folate after the first t_{1/2}.

Furthermore, because pharmacokinetic principles predict that steady state conditions are reached after 5 t_{1/2}, the plateau can be calculated according to the following equation:

\[ C(\text{plateau}) = C(0) + 2 \times \mu \]  

(3)

where \( C(\text{plateau}) \) is the RBC folate concentration at steady state conditions (after 5 t_{1/2}); \( C(0) \) is the RBC folate concentration at baseline, and \( \mu \) is the empiric increase in RBC folate after the first t_{1/2}.

According to pharmacokinetic principles, the dimension of appearance is equal to the division of elimination. Thus, we propose that the elimination kinetics of RBC folate can be calculated on the basis of this t_{1/2} value (see Figure 3) according to the following equation:

\[ C(n) = C(\text{plateau}) - \mu' \times (1 - 0.5^{(n - \text{stoptime})}/(1 - 0.5) \]  

(4)

where \( C(n) \) is the RBC folate concentration after \( n \) t_{1/2}; \( C(\text{plateau}) \) is the RBC folate concentration at steady state; \( \mu' \) is the empiric decrease in RBC folate after the first t_{1/2}, which is equal to \( \mu \) (the empiric increase in RBC folate after the first t_{1/2}); and

stop-time is the point in time when the usage of supplements is stopped.

Application of the kinetic model

For the applicability of the model, the measured and calculated increases in RBC folate concentration would need to be similar. Because the time points of blood sampling in studies 1 and 2 were 8-wk intervals (56 d) and thus close to one t_{1/2} of RBC lifespan (60 d), the results of the 8-wk data points were used for application of the kinetic model. In study 2, which used 800 \( \mu \)g folic acid/d supplements (Table 1), mean RBC folate increased from 654 ± 170 nmol/L to 1166 ± 214 nmol/L during the first t_{1/2}, the mean initial increase (\( \mu \)) was measured as 513 ± 140 nmol/L. After the second t_{1/2} (ie, 16 wk), RBC folate reached a concentration of 1430 ± 243 nmol/L. By applying the kinetic model, the RBC folate concentration after the second t_{1/2} is calculated to reach 1422 ± 262 nmol/L, which is close to the measured concentration of 1430 ± 243 nmol/L. Data from study 1 (Table 1) also give comparable results for the measured and calculated concentration of RBC folate after 16 and 24 wk of supplementation with 400 \( \mu \)g folic acid/d or 416 \( \mu \)g [6S]-5-MTHF/d.

With respect to elimination kinetics, the model predicts a large decrease in RBC folate concentrations in the first t_{1/2}, with subsequently smaller decreases before baseline concentrations are reached after the fifth t_{1/2} (Table 1). We estimate that >8 wk would have to pass after cessation of supplementation with either 800 \( \mu \)g folic acid/d, 400 \( \mu \)g folic acid/d, or 416 \( \mu \)g [6S]-5-MTHF/d for RBC folate concentrations to drop below 906 nmol/L, the RBC folate concentration thought to be optimum for reducing the risk of an NTD-affected pregnancy (1).

DISCUSSION

The kinetic of appearance of folate in RBCs has been investigated in several intervention trials with the administration of...
The initial increase in RBC folate depends on the dose of the folic acid supplementation. When supplementation was stopped, the elimination of RBC folate could not be achieved after 24 wk of supplementation with 400 μg folic acid/d (12) or 113 (14) or 416 (12) μg [6S]-5-MTHF/d. Using data from 2 intervention trials (12, 13), we were able to calculate the biological $t_{1/2}$ for RBC folate and to show its similarity to the $t_{1/2}$ of the RBCs. RBCs have a $t_{1/2}$ of $\approx$60 d, and they incorporate folate only during erythropoiesis (15, 16). The kinetic model estimated that the biological $t_{1/2}$ of RBC folate is $\approx$8 wk (56 d). The measured RBC folate concentrations after the second $t_{1/2}$ (16 wk) of supplementation with either 400 μg folic acid/d, 416 μg [6S]-5-MTHF/d, or 800 μg folic acid/d were similar to the RBC folate concentration calculated with the use of the kinetic model. Therefore, according to pharmacokinetic principles, a steady state condition (ie, a plateau in RBC folate concentration) should be reached after the fifth $t_{1/2}$ (40 wk). The initial increase in RBC folate depends on the dose of the folic acid or folate supplement and on the baseline RBC folate concentration. With knowledge of the initial increase during the first $t_{1/2}$, the plateau of RBC folate can be calculated with the presented formula (3). Furthermore, according to pharmacokinetic principles, the dimension of appearance is defined to be equal to the dimension of elimination. Using a folate depletion model, Sauerberlich et al (19) reported that the decrease in RBC folate concentrations probably reflected the $t_{1/2}$ of the RBC. Thus, we propose that the elimination kinetics of RBC folate can be calculated on the basis of this $t_{1/2}$ value. With knowledge of the plateau of RBC folate concentration and the time at which supplementation was stopped, the elimination of RBC folate could be calculated by using equation 4 (see above).

### TABLE 1

<table>
<thead>
<tr>
<th>Time point</th>
<th>Number of half-life</th>
<th>Appearance $^a$</th>
<th>Elimination $^b$</th>
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<tr>
<td></td>
<td></td>
<td>Study 1</td>
<td>Study 2</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>Measured</td>
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<tr>
<td></td>
<td></td>
<td>Calculated$^2,3$</td>
<td>Calculated$^2,3$</td>
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<tr>
<td></td>
<td></td>
<td>mmol/L</td>
<td>mmol/L</td>
</tr>
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<td>Baseline</td>
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<td>706 ± 239</td>
<td>—</td>
</tr>
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<td>Week 8</td>
<td>1</td>
<td>983 ± 253</td>
<td>1038 ± 214</td>
</tr>
<tr>
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<td>2</td>
<td>1210 ± 217</td>
<td>1322 ± 213</td>
</tr>
<tr>
<td>Week 24</td>
<td>3</td>
<td>1319 ± 237</td>
<td>1445 ± 201</td>
</tr>
<tr>
<td>Week 32</td>
<td>4</td>
<td>1213 ± 312</td>
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</tr>
<tr>
<td>Week 40</td>
<td>5</td>
<td>1230 ± 318</td>
<td>1378 ± 456</td>
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<td>Week 48</td>
<td></td>
<td>—</td>
<td>1378 ± 456</td>
</tr>
<tr>
<td>Week 56</td>
<td>1</td>
<td>960 ± 244</td>
<td>1003 ± 281</td>
</tr>
<tr>
<td>Week 64</td>
<td>2</td>
<td>825 ± 229</td>
<td>816 ± 234</td>
</tr>
<tr>
<td>Week 72</td>
<td>3</td>
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<tr>
<td>Week 80</td>
<td>4</td>
<td>724 ± 231</td>
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<td>Week 88</td>
<td>5</td>
<td>707 ± 233</td>
<td>652 ± 236</td>
</tr>
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<td></td>
<td></td>
<td>654 ± 170</td>
<td>—</td>
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</table>

$^a$ All values are arithmetic $\bar{x} \pm SD$. The kinetic model is based on pharmacological principles assuming that the dimension of appearance is equal to the dimension of elimination. The 2 trials were reported in references 12 and 13.

$^b$ The appearance of folate in RBCs follows the equation: $C(n) = C(0) + \mu \times (1 - (1 - 0.5^{n-t_{1/2}}) / (1 - 0.5))$, in which $C(n)$ is the RBC folate concentration after $nt_{1/2}$; $C(0)$ is the RBC folate concentration at baseline, and $\mu$ is the empiric increase in RBC folate after 8 wk (1 $t_{1/2}$).

$^c$ The elimination kinetics of RBC folate is calculated on the basis of $t_{1/2}$ according to the formula $C(n) = C(\text{plateau}) - \mu' \times (1 - 0.5^{n-stoptime})/(1 - 0.5)$, in which $C(n)$ is the RBC folate concentration after $nt_{1/2}$; $C(\text{plateau})$ is the RBC folate concentration at steady state, $\mu'$ is the empiric decrease in RBC folate after 8 wk (1 $t_{1/2}$), which is equal to $\mu$, and stoptime is the point in time when the use of supplements is stopped.

Because RBCs have a lifespan of $\approx$120 d, incorporate folate during erythropoiesis, and release it only during hemolysis, RBC folate serves as a long-term indicator of folate status (15, 16). Folates seem to be retained through the lifespan of an RBC, probably by protein binding (15). Folate is considered to be stable and inactive in RBCs. Because of the oxidative environment, one might expect folate that had accumulated in RBCs to undergo changes in concentration or physiologic form during the $\approx$120-d lifespan of the RBCs. If folate underwent transformation or eventual diffusion out of the RBCs, either of those processes would follow passive mechanisms and thus happen in a constant dimension. An eventual, constant—but rather minimal—decrease in RBC folate due to degradation or diffusion would not influence its $t_{1/2}$. With the use of an in vivo folate kinetic model, Stites et al (20) reported long-term folate stability. Their study showed a slow turnover of the whole-body folate pool and a mean residence time for in vivo folate molecules of $\geq$100 d (20).

Even though RBC folate serves as a marker for long-term folate status, RBCs are not “supplying” tissues with folate. However, RBC folate was reported to correlate with liver folate concentrations (21). RBC folate concentrations were used as a marker of folate status for the determination of the relation between folate status and the risk of an NTD-affected pregnancy (1); however, plasma is the provider of folate during pregnancy. Plasma folate reflects immediate changes in folate intake and turnover (15).
In a public health strategy that was intended to reduce the occurrence of NTDs, some countries—e.g., the United States (23), Canada (24), and Chile (25)—implemented mandatory fortification of food with folic acid. After folic acid was added to grain products, a decrease in the occurrence of NTDs by up to 54% was observed in these countries (26–28). In the United States, a 19% decrease in NTD prevalence was observed after grain products were fortified with folic acid (26) at an amount intended to provide an additional 100 μg folic acid/d (23). However, the increase in daily intake of folic acid was higher than predicted, at ≈200 μg/d (29, 30). An even greater reduction in the prevalence of NTDs was observed in Chile. The incidence of the 2 forms of NTDs, anencephaly and spina bifida, decreased by 42% and 51%, respectively (28). In Chile, the implementation of food fortification with folic acid was intended to provide an extra 400 μg folic acid/d (25). However, 2 studies using periconceptional folic acid supplementation of 4000 and 800 μg folic acid/d showed a decrease in NTDs of 72% and 100%, respectively (4, 5). An increase in the amount of folic acid added to fortified food is thus not supported because of the possible negative side effects when the general population is chronically exposed to a large amount of folic acid. Health authorities are concerned about the possibility that some persons, especially those who also use vitamin supplements, may exceed the tolerable upper intake level (UL) of folic acid (30, 31). Folic acid intake above the UL of 1 mg/d can delay the appearance of the hematologic symptoms of a vitamin B-12 deficiency (23, 32), whereas the consumption of 65-5-MTHF may not do so. Because only 10–47% of women are using periconceptional folic acid supplementation (33–37) and because a relatively low amount of folic acid has been added to fortified food, the greatest possible potential of folic acid in the prevention of NTDs has not yet been explored by public health measures.

With respect to the elimination of folate, we hypothesize that the elimination kinetics are equal to the dimension of the appearance of folate in RBCs according to pharmacokinetic principles. Currently, a long-term intervention trial to test this hypothesis is underway. If the elimination of RBC folate can be predicted according to a particular amount of supplemented folate or folic acid, we would like to discuss the possibility and eventual effect of adding folate or folic acid to oral contraceptives (OCs). One advantage of fortifying OCs would be the reduced NTD risk during OC use. Even women who are using OCs may get pregnant as a result of inconsistencies or mistakes in taking the OCs. Use of OCs is common, especially in young women. Sixty-seven percent of the women participating in our 2 intervention trials were using OCs (12, 13). Compared with that percentage, only 10–47% of the women in several studies (33–37) reported using periconceptional folic acid supplementation, despite promotional campaigns. After cessation of fortified OC use, eg, when a woman is seeking to become pregnant, the RBC folate concentration may remain above the “protective concentration” of 906 nmol/L (1) for a certain amount of time. This period until the RBC folate concentration drops to <906 nmol/L could be estimated. In addition, upon ceasing OC use, a woman would have protective concentrations of folate in case she quickly became pregnant. Thus, having taken folate during OC use, a woman would not need to wait ≥12 wk (12) before her folate concentration reached the protective concentration but rather would be protected even during early supplementation with ≥400 μg folate or folic acid/d. Thus, the addition of folate or folic acid to OCs may be considered an alternative strategy. However, further studies are needed to investigate the elimination kinetics of folate in different tissues.

In conclusion, this kinetic model presents an estimation of the 1/2 period of RBC folate, which is a marker of long-term folate status and is related to a woman’s risk of an NTD-affected pregnancy. The biological 1/2 period for RBC folate concentration was calculated to be >8 wk. An application of pharmacokinetic principles indicates that steady state conditions for RBC folate should be reached after the fifth 1/2 period, or 40 wk. These pharmacokinetic principles also assume that the appearance rate is equal to the elimination rate. Thus, elimination kinetics of RBC folate can be calculated on the basis of this 1/2 period. The period of time during which RBC folate concentrations would remain above the RBC folate concentration related to the lowest NTD risk (ie, 906 nmol/L) was calculated to be >8 wk. The elimination kinetics of folate may be of special interest for new prevention measures with respect to NTD prevention.

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The authors’ responsibilities were as follows—KP and YL: shared authorship; KP: the original concept of the kinetic model and the derivation of the kinetic formula; YL: wrote the manuscript draft; KP, YL, SB, and RP-L: the conduct of the 2 intervention studies; and SB and KP: assisted in manuscript preparation. None of the authors had any personal or financial conflict of interest.

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