Erythrocyte Folate and Its Response to Folic Acid Supplementation Is Assay Dependent in Women1,2

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ABSTRACT Optimizing folate status requires continued monitoring of erythrocyte (RBC) folate and folate intake. The accuracy of RBC folate assays remains a concern. Therefore, we measured RBC folate with 4 different assays, examined the interassay correlations, and compared RBC folate with folate intake as measured by an abbreviated folate-targeted food/supplement screener. The screener had 21 questions (19 diet, 2 supplement) and measured usual and customary intakes of dietary folate equivalents (DFEs). Our design was a 4 × 2 × 2 factorial, 4 assays in pregnant and nonpregnant women before and after each group received a folic acid supplement (1814 nmol/d) for 30–60 d. Folate assays included L. casei, chemiluminescence, GC-MS, and radioassay (RA). Baseline RBC folate levels ranked low to high by assay (mean ± SE) were as follows: 1155 ± 44 nmol/L (L. casei) < 1390 ± 43 nmol/L (chemiluminescence) < 1531 ± 39 nmol/L (GC-MS) < 1727 ± 56 nmol/L (RA) (P < 0.0001). Supplementation raised RBC folate levels (mean ± SE) as follows: 138 ± 63 nmol/L (chemiluminescence) < 267 ± 64 nmol/L (GC-MS) < 285 ± 75 nmol/L (L. casei) < 351 ± 87 nmol/L (RA). Pregnant women had higher RBC folate than nonpregnant women using chemiluminescence and RA. Interassay correlations (r) ranged from 0.4679 to 0.8261 (P < 0.0001). Correlations of RBC folate with folate intake ranged from 0.2676 to 0.4622 (P < 0.0004). We conclude that RBC folate levels are assay dependent, as is the definition of optimized status; there continues to be a need for an accurate assay of RBC folate. RBC folate correlated with total folate intake using a folate-targeted food/supplement screener. J. Nutr. 135: 137–143, 2005.

KEY WORDS: ● RBC folate ● analytical method ● low-income women ● folate-targeted food screener

Adequate intake of folate is important to minimize the risk of neural tube defects (NTDs)1 (1,2), cardiovascular disease (3), and some cancers (4). An adequate folate intake by women of child-bearing age is especially important because it can raise RBC folate levels, and has been shown to reduce the risk of NTDs by ~70% (5). The RBC folate level is rated a good biomarker for nutritional status of this important nutrient because of its correlation with liver folate, a significant tissue store (6). Cell accumulation of folate may be key to ameliorating NTD susceptibility with increased folate intake (7). Therefore, the strength of the relations between folate intake and biomarkers of folate status such as RBC folate is of considerable interest.

Women who fall below 185% of the Federal Poverty Level (FPL ≤ 1.85) are an important target population group for improving folate status. They may not fully appreciate the importance of folate (8). The correlation between folate intake and RBC folate concentrations has not been adequately studied in this population; we were prompted to measure their folate status using their RBC folate levels and to examine its correlation with their folate intake. The intake was measured with a new abbreviated folate-targeted food/supplement frequency screener that was developed for this study.

Plasma and serum folate concentrations are commonly accepted to reflect recent dietary folate intake. Although erythrocyte (RBC) folate is considered to be a better indicator of body stores and hence nutritional status (9), there is considerable uncertainty about the reliability of the analytical methods for RBC folate (10,11). An interlaboratory comparison of popular analytical methods for folate revealed large differences among laboratories analyzing a common set of serum and whole-blood specimens (10). Results of the interlaboratory comparison prompted the recent development of promising new hyphenated methods that included LC-MS and LC-MS-MS for plasma and serum folate (12–15) and GC-MS for RBC folate (16–18). Like the microbiologic assay methods, the new hyphenated methods can be internally and externally standardized.
Here we present a feasibility study in which we compared a GC-MS assay to 3 other common assays for RBC folate. Also, we examined the correlation between RBC folate levels and folate intake as measured by an 18-item folate-targeted food/supplement Dietary Folate Equivalents (DFE) screener developed for this study. The time required to complete the DFE screener (6–12 min) makes it an attractive alternative to longer FFQs and for epidemiologic investigations focused on folate status. If further research demonstrates the validity of the DFE screener when it is self-administered, as opposed to administered by clinic staff via interview, this instrument may prove useful as a classification tool for clinical settings.

SUBJECTS AND METHODS

The University of California Davis Institutional Review Board approved the study, and it was conducted according to Good Clinical Practice guidelines and the Declaration of Helsinki, version 1989. Informed consent was discussed and obtained from each woman before study participation. Women were excluded for any history of serious medical conditions or use of medications that would interfere with folate metabolism (dilantin, phenytoin, primidone, metformin, sulfasalazine, triamterene, or metotrexate). Intermittent users of a multivitamin supplement (1–2 times/wk) were included, whereas prenatal-vitamin supplement users were excluded. All pregnant women were enrolled at the time of their first prenatal visit and were not taking any prenatal vitamins.

Subject enrollment. Enrollment occurred from March through September 2003. Pregnant women (n = 29), and nursing or non-pregnant low-income women (n = 39) who were at least 18 y old, and who were eligible or who had begun the Women, Infants, and Children (WIC) program during the preceding 2 wk, were enrolled in this study. All study instruments (consent, 1-page folate-targeted food screener) were translated into Spanish, back translated into English, and administered by bilingual research staff.

The pregnant women were enrolled primarily from 6 local community clinics in Sacramento, CA. The clinics were providers of the county Comprehensive Perinatal Services Program (CPSPP) and Medi-Cal programs. CPSPP and Medi-Cal are statewide government programs that provide comprehensive prenatal care to low-income women. To be eligible for these programs, a family’s income must be ≤185% of the Federal Poverty Level (FPL ≤ 1.85), i.e., gross monthly income of $1400–$5000 for a family unit of 1 to 8 persons, respectively. We targeted pregnant women who were scheduled for their first obstetrics appointment because they were most likely to have not started using supplements. We also targeted women who were newly recruited into the WIC program. WIC is a federal grant program that provides vouchers for supplemental foods, nutrition education, and prenatal counseling to low-income women. WIC food vouchers are special checks for purchasing health-promoting foods such as milk, juice, eggs, peanut butter, and so forth. The WIC authorized food list of July 2003 targeted foods rich in vitamins A and C, protein, and fiber, but not folate. Women enrolled in this federal program have been shown to improve their nutritional status and pregnancy outcome (19,20). Community healthcare practitioners refer pregnant women to the WIC program to initiate nutrition intervention.

The nonpregnant women were enrolled from a local Woman’s Center. This Center is a nonprofit organization in Sacramento, CA that serves low-income women and their children by providing them a daily meal, and referrals to local state agencies for food, housing, and shelter.

Clinical visits. Women enrolled at the 1st clinic visit (baseline values) were questioned about their general medical history, dietary habits, length of time on WIC, and demographics. Each woman completed a 1-page folate-targeted food/supplement intake screener, and blood was drawn as described below. Pregnant women began taking a standard over-the-counter prenatal vitamin supplement (800 μg or 1814 nmol folic acid/tablet) as prescribed by their healthcare provider. All nonpregnant women received one bottle of 100 prenatal vitamin tablets (with the same amount of folic acid/tablet, Longs Drug Store Brand) to match the pregnant group. In addition to folic acid, each tablet also supplied vitamin B-12, 4 μg (3 nmol); vitamin B-6, 2.6 mg (11 μmol); and iron, 27 mg (490 μmol). All women were instructed to take 1 prenatal tablet/d until their 2nd clinic visit. A $25 gift certificate to a local supermarket (for food only) was given to each woman at the end of the visit.

The 2nd clinic visit occurred 30–60 d after supplementation first began. Only 3 of 38 nonpregnant women were lactating at this visit. All women again completed the 1-page folate-targeted food/supplement intake screener, and blood was drawn again from each woman as described above. All women were queried about compliance with supplement use, i.e., how often they took/missed taking the supplement. The overall compliance was determined from the 1-page folate-targeted food/supplement intake screener, interviews, and queries. Pregnant women were asked about their start date for receiving WIC benefits. At the end of the visit, another $25 gift certificate to a local supermarket (for food only) was given to each woman.

Folate-Targeted Semiquantitative Block DFE screener. The semiquantitative Block Dietary Folate Equivalents (DFE) screener was designed to measure usual and customary intake of DFE and was optimized for use in low-income populations.1 The questions on the screener were identified by examining the food records reported by individuals ≥17 y old in the National Health and Nutrition Examination Survey (NHANES) 1999–2000. To develop the screener, all 4368 food items and beverages in that dataset were divided into 152 food groups (questions). Each question represented a similar type of food item (for example, pinto, red, black, or refried beans, separately, or as part of another food such as a “burrito,” were coded together as beans). The folate contributed as DFE by each food in the NHANES dataset was calculated by multiplying, for each NHANES respondent, the mass of the food consumed by its folic acid concentration, and then summing across all of the respondents. To calculate DFEs from the NHANES data, the estimated synthetic folic acid (SFA) content of each food was multiplied by 1.7, the bioavailability correction factor provided by the Institute of Medicine (21). Food groups (questions) were then ranked according to their contribution to total DFE intake; 19 questions, representing foods contributing the top 60% of U.S. DFE intake, were included in the Block DFE screener. The total DFE consumed by each woman enrolled in our study was calculated as the cumulative sum of DFEs contributed by each food group on the screening questionnaire. Folate values for the questions on the DFE screener were identified by analysis of the USDA Nutrient Database for Standard Reference, Release 15 (August, 2002). Synthetic folic acid values used for the computation of DFEs from vitamin supplements were identified by reference to label claims on commonly available vitamin supplements.

Blood drawing and analysis. Blood was drawn from each woman into 4 separate 4-mL tubes that contained spray-dried K2-EDTA and one SST tube for vitamin B-12. The tubes were inverted gently to prevent clotting. All EDTA tubes for whole-blood folate were promptly wrapped in aluminum foil (to exclude light) and immediately stored on dry ice. One EDTA tube and the SST tube were stored at −20°C during transit (~2 h) to the University of California Davis, Sacramento Medical Center (UCDMC) Pathology Department Laboratory where complete blood count, packed cell volume (PCV), and serum vitamin B-12 (SimulTRAC Radioassay Kit, Vitamin B-12[57Col]/Folate[57]) from ICN Diagnostics) measurements were made. Also, one frozen tube of whole blood was analyzed for folate using chemiluminescence technology (Bayer Diagnostics ADVIA Centaur, Bayer Corporation Diagnostics Division) at the UCDMC Pathology Department Laboratory. The remaining 3 EDTA tubes were stored on dry ice during transit (~2 h) to the laboratories where they were stored at 70°C for RBC folate analysis using GC-MS (16–18), Lactobacillus casei (22), and radioassay (RA; SPNB Dual Count 125I, Diagnostic Products) methods. The L. casei and RA assays were conducted at the UCD-Clinical Nutrition Research Unit (UCD-CNRU). The Chemiluminescence, L. casei, and RA assays were performed on whole-blood lysates (prepared with ascorbic acid). Folate concentrations of whole blood were divided by the PCV (fraction) and expressed as nmol folate/L erythrocytes. A pool of freshly drawn blood was promptly divided into small aliquots and...
stored at −70°C; aliquots from the pool were analyzed with each batch of study samples to serve as a control monitor for day-to-day variation. Finally, all RBC folate assays were performed within 3 wk from the date the blood was drawn.

Calculations and data analysis. The data were checked for normality, and descriptive statistics were calculated for each response variable. The data were analyzed by repeated-measures ANOVA. The 3-way interaction (assay × supplementation × pregnancy status) was not significant; thus, only main effects (Table 1) and the 2-way interactions, assay × supplement and assay × pregnancy status (Figs. 1, 2) are presented. Regression analysis of RBC folate levels by the 4 assay methods and by folate intake were also calculated. All statistical analyses were conducted with StatView 5.0.1 software program (Abacus Concepts).

RESULTS

Of the 30 pregnant women, 24 completed the study as did 29 of the 38 nonpregnant women. Those who did not complete the study were lost to follow-up for not keeping their scheduled clinic appointments, termination of pregnancy, or moving out of the area. Of the 30 pregnant women, 6 were Caucasian, 8 were African American, 14 were Latina Hispanic, 2 were other (Native American); 17 were single and 13 were married; 4 lived alone, 24 lived with others, and 2 were homeless. Of the 38 nonpregnant women, 8 were Caucasian, 15 were African American, 13 were Latina Hispanic, and 2 were other (Asian); 26 were single and 12 were married; 6 lived alone, 28 lived with others, and 4 were homeless. The pregnant and nonpregnant women had similar education levels, 11 ± 3 y (mean ± SE), also similar to that of women participating in the NHANES III (11.3 ± 0.06 y, mean ± SD) for which the data were collected between 1988 and 1994 (23).

Other characteristics of the study participants are summarized in Table 1. The pregnant women were younger than the nonpregnant women by approximately a decade. The pregnant women were in wk 18 ± 2 of gestation and they were currently not receiving WIC benefits. The pregnant group had fewer children (P = 0.0140) than the nonpregnant group, but the difference would likely disappear when the pregnant group was as old as the nonpregnant group.

As expected, the pregnant women had a lower PCV (P < 0.0001), RBC count (P < 0.0001), blood hemoglobin (P < 0.0001), and plasma vitamin B-12 levels (P = 0.0749) compared with nonpregnant women. Also, the pregnant women had a higher white blood cell (WBC) count (P = 0.0073) than the nonpregnant women. Folic acid supplementation did not significantly affect PCV, WBC count, RBC count, hemoglobin, or plasma vitamin B-12. The study population was normal.

RBC folate levels were dependent on assay method and supplement (Table 1, Fig. 1). The overall mean concentrations (main effects of assay method ranked low to high) were as follows: 1278 ± 39 nmol/L by L. casei < 1450 ± 32 nmol/L by chemiluminescence < 1646 ± 34 nmol/L by GC-MS < 1877 ± 46 nmol/L by RA (P < 0.001). In addition, the magnitude of the increases in RBC folate (nmol/L) in response to supplementation (ranked low to high) were as follows: 138 ± 63 nmol/L by chemiluminescence < 267 ± 64 nmol/L by GC-MS = 285 ± 75 nmol/L by L. casei < 351 ± 87 nmol/L by RA. RBC folate levels were dependent on assay method and pregnancy status (Table 1, Fig. 2) with pregnant women having a higher RBC folate concentration only when analyzed by chemiluminescence and RA.

The Block DFE screener had 21 questions (19 diet, 2 supplement) and measured usual and customary intake of DFEs. It included the 19 questions that account for 60% of total folate intake as DFEs in the U.S. diet, and 2 questions concerning vitamin supplements. The women enrolled in our study completed the screener in 6–12 min.

### Table 1

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<th>Characteristics, hematology, erythrocyte folate, and folate intake of women in the study¹</th>
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¹ Values are means ± SEM.
² The estimated SFA content of each food was multiplied by 1.7, the bioavailability correction factor provided by the IOM (21).
The intake of natural food (NF) folate was the same for the pregnant and nonpregnant women (Table 1). Because the pregnant group had a higher intake of SFA from fortified foods (breakfast cereals) than the nonpregnant group ($P = 0.0269$), there was a trend ($P = 0.0644$) for them to have a higher intake of total food folate (NF + SFA). Differences in the food (NF + SFA) intake did not differ between the baseline and supplementation periods ($P = 0.5742$).

The overall compliance with taking the folic acid supplement was as follows: 64% of women reported taking the supplement every day; 21% reported taking it 4–6 d/wk; 7.5% of women depended on daily supplementation; and 21% reported taking it 4–6 d/wk; 7.5% reported taking it 1–3 d/wk; and 7.5% reported that they had stopped taking it 30–60 d before giving birth. This level of compliance resulted in the supplement providing 596 μg/d (1360 nmol/d) SFA supplements for 30–60 d. Baseline levels and the increase upon supplementation were assay dependent.

The interassay correlation coefficients ($r$) for RBC folate ranged from 0.4679 (GC-MS vs. chemiluminescence) to 0.8261 ($L. casei$ vs. RA) (Fig. 3). The $r$-value for RA vs. GC-MS was 0.7063, the 4th highest of 6.

Relations between RBC folate levels (using 4 analytical methods) and folate intake as DFEs ranged from 0.2676 with chemiluminescence to 0.4622 with RA (Fig. 4). The $r$-value for folate intake vs. GC-MS-assayed RBC folate was 0.4343 ($P < 0.0001$), the 3rd highest of 4. Although the $r$-values were significant, only 16% (a modest portion) of the variation in a dependent variable was explained by the independent variable when $r = 0.40$.

**DISCUSSION**

Because RBC folate reflects intracellular and tissue folate stores, it was chosen as the preferred indicator of folate nutritional status in establishing the new dietary reference intakes (21). At the same time, there are concerns over the accuracy of existing analytical methods for RBC folate (10,11) and only a few groups (16,17,25–27) report within-laboratory comparisons of several RBC folate assays. Therefore, we used 4 different methods to measure RBC folate levels in small populations of pregnant and nonpregnant women (FPL = 1.85) before and after each received a daily supplement of 1814 nmol folic acid for 30–60 d. Also, we measured folate intake with a 1-page folate-targeted food/supplement frequency screener designed specifically for our study (Block DFE screener), and determined whether folate intake was correlated with RBC folate.

Maternal RBC folate > 906 nmol/L, measured with Quantaphase IR Folate Radioassay (5), is considered optimal for prevention of folate-responsive neural tube defects (28). When we used chemiluminescence, GC-MS, and RA methods, only 5, 1, and 3 women, respectively, had RBC folate < 906 nmol/L. However, when we used $L. casei$, 24 women had RBC folate < 906 nmol/L. A goal of Healthy People 2010 is to increase RBC folate of nonpregnant women to 500 nmol/L (29) and only the $L. casei$ assay identified women ($n = 3$) with RBC folate < 500 nmol/L in our study. Another research group (30) used the $L. casei$ assay to determine RBC folate status in a similar population of women residing in California postfortification and not taking any supplements. Their respective levels for socioeconomically disadvantaged and advantaged women were 1172 ± 342 nmol/L and 1387 ± 329 nmol/L, levels similar to ours. Our mean RBC folate levels and those of Caudill et al. (29) both far exceeded 362 nmol/L (9), a concentration that is widely considered an acceptable cutoff value.

Only 1 subject in our present study had an RBC folate level < 362 nmol/L; the lowest individual RBC folate values were 333 nmol/L by $L. casei$, 653 nmol/L by chemiluminescence, 806 nmol/L by RA, and 894 nmol/L by GC-MS. Of the women in our study, 65% had RBC folate > 906 nmol/L, which compares well with the value of 78% reported by another research group (30). Using the $L. casei$ method, the mean RBC folate concentration in the Framingham Offspring Cohort study after fortification was 1020 nmol/L (31), which agreed with $L. casei$-measured baseline values in Table 1. Our 30- to 60-d supplementation period was chosen to equal or exceed the recommended 4-wk period of folic acid use before conception to improve folate status (21,32,33) and/or the 4-wk period used in another study (34).

Since 1998, women capable of becoming pregnant have been advised to consume 907 nmol folic acid/d from fortified foods and supplements, in addition to dietary folate (21). The
fortified foods and supplements together provided the pregnant and nonpregnant groups with 939 ± 55 and 950 ± 64 μg/d (2129 ± 125 and 2154 ± 145 nmol/d) of SFA, respectively (Table 1). These quantities of SFA almost matched those recommended since 1998 (21).

Participants in the present study consumed ~600 μg/d (1360 nmol/d) supplement (SFA) and experienced (mean) increases in RBC folate of 138–351 nmol/L, depending on the assay used. Had we doubled the duration of the supplementation period to match the ~120-d lifespan of RBCs, and/or increased the amount of the SFA supplement, we might have achieved a higher rise in RBC folate in response to supplementation. The rise in RBC folate remained linear with no evidence of saturation as total folate intake increased irrespective of the assay method (Fig. 3); therefore, the plateau level of RBC folate remains unknown. Further investigation is warranted to establish a reference range for RBC folate levels to prevent anemia and another to minimize the risk of more serious defects (35).

The protein binding folate assays (chemiluminescence and RA) are facile compared with the \textit{L. casei} and GC-MS assays, which require several steps in sample preparation (16,17,36). Among the protein-binding assays, the magnitude of the increases in RBC folate (nmol/L) upon supplementation had a lower \(P\)-value with chemiluminescence (\(P < 0.0171\)) than with the RA assay (\(P < 0.0001\)), whereas the magnitude of the rise with the \textit{L. casei} and GC-MS assays did not differ (Table 1, Fig. 1). When the protein-binding assays were employed, pregnant women had higher RBC folate levels than nonpregnant women (Table 1, Fig. 2), suggesting that these assays may be hormone sensitive. Finally, RBC folate, as determined by \textit{L. casei} was correlated with the concentration measured by GC-MS with an \(r = 0.7185\), the 4th highest of 6 (Fig. 3, Panel B). Therefore, it seems that the \textit{L. casei} and GC-MS methods may offer hope for accurate RBC folate determinations that can serve as guides to optimal folate status.

The RBC folate as determined by RA was correlated with the concentration measured by GC-MS with an \(r = 0.7063\) (Fig. 3, Panel C); this did not differ from the \(r = 0.7010\) value reported by another group (26) who compared a number of commercially available protein-binding assay kits.

The Block DFE screener was effective in assessing dietary intake of folate because the DFEs measured by the screener were positively (and modestly) correlated with the results of all 4 folate assay methods (\(r = 0.26–0.46\)) and the \(r\)-values were significant (\(P < 0.0001\)) for all measures (Fig. 4). Yen et al.
found a similarly modest correlation ($r = 0.354$, $P = 0.06$) when they compared folate intake measured by 7 d of “focused recalls” with plasma folate levels. They also found no significant correlations between the Fred Hutchinson Cancer Research Center FFQ and plasma folate (37). Comparisons between correlations reported by Yen et al. (37) and those presented here for the Block DFE screener should be interpreted with caution, because the 2 dietary instruments have different purposes. The instrument of Yen et al. (37) was designed to measure short-term folate intake and to compare it with plasma folate, whereas the Block DFE screener was designed to measure usual, long-term folate intake and to compare it with RBC folate, a good biomarker of long-term folate nutritional status.

The Block DFE screener was designed to capture foods contributing the top 60% of folate intake (as DFE) in the United States, and was not intended to capture a point estimate that would reflect 100% of folate intake. Longer FFQ, however, are often designed to capture 80–90% of nutrient intake. The Block DFE screener produces lower estimates than a full-length FFQ, or than the truth, by design. The purpose is to create an instrument that would rank subjects well, while being brief. Block et al. (38,39) showed that shorter questionnaires such as the DFE screener rank subjects similarly to full-length FFQ.

We conclude that RBC folate and its response to folic acid supplementation was assay dependent; thus, method-specific RBC folate ranges may be necessary in evaluating published data at this time and setting cutoff values. Further comparisons among assay methods are warranted to identify the method that most accurately determines the actual RBC folate value. The folate screener was effective in assessing dietary folate intake. The adequacy of the folate nutritional status (of our subjects) reflects the fortified diets.

**LITERATURE CITED**


