Determination of unmetabolized folic acid in human plasma using affinity HPLC[1–5]

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ABSTRACT

Background: Folic acid (FA) fortification of food created the need to determine whether fortification elevated concentrations of unmetabolized FA in plasma and whether this form of the vitamin in blood is associated with adverse health outcomes.

Objective: The objective of this research was to devise a simple, rapid method for the measurement of unmetabolized plasma FA in epidemiologic studies.

Design: We previously used the affinity/HPLC with electrochemical detection method to measure folate distribution in human plasma and red blood cells (RBCs). We modified this method with the inclusion of synthetic ethyltetrahydrofolate as an internal standard and with the use of 2 affinity columns connected in parallel to the analytic column through a switching valve to allow one column to be loaded while the other column was eluted into the analytic column.

Results: We identified FA and 5-methyltetrahydrofolate (5-mTHF) by retention time and characteristic response across the channels of the electrochemical detector. Limits of detection were 0.034 pmol for 5-mTHF and 0.027 pmol for FA per injection, and the recovery was 92.2% (5-mTHF) and 98.9% (FA). CVs for samples were 8.1% for (within day) and 6.8% (between day) for 5-mTHF and 3.2% (within day) and 5.9% (between day) for FA. Total folate with the use of this method correlated highly ($r^2 = 0.98$, $P < 0.001$) with values from the microbial assay. The run time for the method was 30 min per sample. Researchers can use this method with longer run times to measure the distribution of folate forms in RBCs.

Conclusion: This updated method allows efficient analysis of folate forms in human plasma and tissues without the loss of sensitivity or precision. *Am J Clin Nutr* doi: 10.3945/ajcn.111.013433.

INTRODUCTION

To decrease the incidence of neural tube defects, the US Food and Drug Administration mandated fortification of all enriched cereal-grain products with folic acid (FA) by January 1998 (1). This policy was associated with a decrease in neural tube defects (2) and stroke-related mortality in the United States (3). In addition, fortification was associated with the virtual elimination of folate deficiency and a decrease in plasma homocysteine concentrations (4–7).

FA, the form of folate that manufacturers use for fortification, is a synthetic form of the vitamin that requires reduction to tetrahydrofolate (THF) before incorporation into the active cellular folate pool. In humans, this reduction has limited capacity, and when people take excess FA (ie, >200 μg), elevated amounts of unmodified FA appear in the circulation (8, 9). Eventually, the body converts much of this FA into THF and the peripheral tissue takes up the THF and incorporates it into cellular folate.

A recent study from our group has shown that in women aged >60 y, plasma FA concentrations have an inverse relation to natural killer cell cytotoxicity (10). This finding is consistent with recent suggestions that high concentrations of unmetabolized FA in the circulation are potentially harmful (11, 12). However, because of the lack of suitable methods to measure unmetabolized FA in populations, research in this area has been limited. We describe a modification of our method that combines affinity/HPLC with electrochemical detection for folate analysis (13) to measure unmetabolized FA concentrations in plasma for population studies.

MATERIALS AND METHODS

Preparation of samples

We selected plasma samples for the assays from the archived plasma pools we used in our laboratory. We thawed these samples at least once for various measurements but otherwise kept them at −80°C. We mixed the plasma samples (0.2 mL) in a cold ice bath with 1.2 mL of 50 mmol potassium tetraborate/L that contained 1% sodium ascorbate (pH 9.0). We added 20 pmol of synthetic ethyltetrahydrofolate (eTHF) to each plasma sample as an internal standard. We vortexed the mixture and boiled it for 30 min.

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[2] Presented at the conference "NHANES Monitoring of Biomarkers of Folate and Vitamin B-12 Status: a Roundtable Review," held in Rockville, MD, 15–16 July 2010.

[3] The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Center for Health Statistics, the Centers for Disease Control and Prevention, the National Institutes of Health, the US Department of Health and Human Services, the US Department of Agriculture, or the authors' affiliated institutions.

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We then kept the mixture in the dark overnight at 4°C. Before HPLC analysis, we filtered the samples with a 0.22-μm filter and used the filtrate for analysis or kept it at −80°C until the analysis. We extracted folate from red blood cells (RBCs) with the use of a method similar to the one we described for plasma, except that we added Triton X-100 (0.2%) to the extraction buffer.

Affinity/HPLC

The affinity/HPLC system consisted of *1*) 2 affinity columns that we connected in parallel through a Rheodyne 7126 automatic, 10-port/2-position switching valve (Rohnert Park, CA) to an analytic column (Betasil Phenyl 250 × 4.6 mm; Keystone Scientific, Bellefonte, PA); *2*) a Waters model 515 pump (Milford, MA); *3*) an Agilent 1100 HPLC system (Santa Clara, CA) with ChemStation software and a quaternary pump with vacuum degasser and cooled liquid sampler with an automatic injector; and *4*) an ESA CoulArray electrochemical detector (Chelmsford, MA) with a Model 6210 4-sensor cell. We controlled all of the operations except the Waters pump with the use of the Agilent 1100 software.

An outline of the system is provided in **Figure 1**. Pump A (Waters), which connected to the Agilent injection valve, delivered mobile phase C (0.05 mmol potassium phosphate/L, pH 7.0, which contained 5% acetonitrile) at a constant rate of 0.35 mL/min. The purposes of this step were equilibration; sample loading into the affinity columns; and subsequent washing to rid the column of nonfolate material, particularly the ascorbate that we used for sample preparation, which interferes with the electrochemical detection. Pump B (Agilent) delivered an acetonitrile gradient at acid pH through the second affinity column and the analytic column. The purpose of this process was to elute the folate from the affinity column and subsequent fractionation by the analytic column for electrochemical detection.

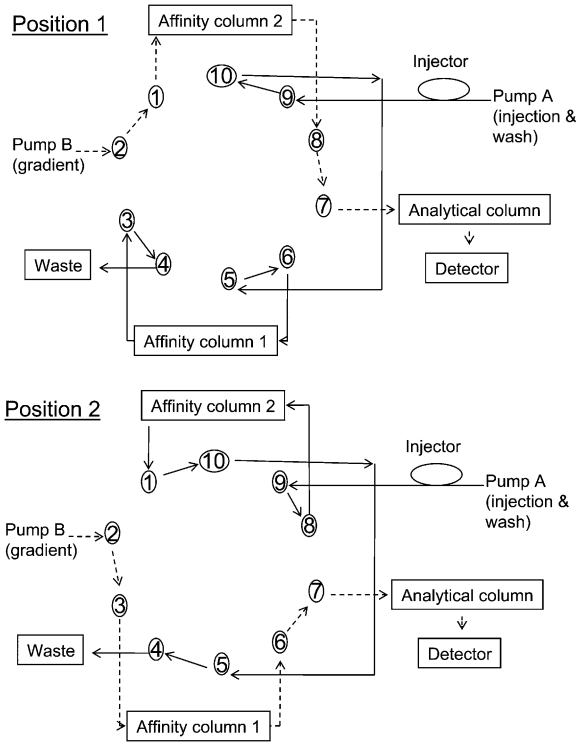

FIGURE 1. Flow diagram for the 2 online affinity columns connected through a 10-port, 2-position valve to an analytic column and a CoulArray detector (ESA, Chelmsford, MA) to evaluate folate forms. When the valve switch is in position 1, affinity column 1 is loaded with the sample and washed while affinity column 2 is eluted into the analytic column. These processes are reversed when valve switch is in position 2.

The gradient comprised mobile phase A, whose buffer was 28 mmol dibasic potassium phosphate/L and 60 mmol phosphoric acid/L (pH 3.0), and mobile phase B, which used the same buffer as mobile phase A with 20% acetonitrile. The elution schedule is presented in **Table 1**. We set the cell potentials for the electrochemical detector at 0, 300, 500, and 600 mV for channels 1–4. We automatically set the detector at zero 0.25 min into the run. We performed the cell cleaning function at 700 mV per cell for 30 s at the end of each sample. Except for the Waters pump, we controlled the Rheodyne switching valve and the ESA on and off positions through contact switches in the ChemStation software.

The system delivered the injected sample (900 μL) into affinity column 1 (Figure 1) and subsequently washed it with mobile phase C until a signal from the ChemStation caused the Rheodyne valve to switch to position 2. While the system loaded affinity column 1 with the sample, it eluted the folate in affinity column 2 into the analytic column and then performed fractionation and electrochemical analysis. The Rheodyne valve was switched to position 2 to reverse the procedures for the 2 affinity columns. Run time and injection time totaled 30 min per sample.

We applied the same principle of 2 affinity columns and electrochemical detection for analysis of RBC folate, which comprises folate forms that have different levels of polyglutamation. In this case, the run time was longer (50 min) to allow for the elution and separation of these polyglutmatyl folates (**Table 2**).

Preparation of folate standards

We synthesized eTHF from THF as follows: we added 1 mg THF (Sigma-Aldrich, St Louis, MO) to a tube that contained 0.05 mL acetaldehyde and 0.02 mL mercaptoethanol. We kept this solution on ice and then added 25 mg solid potassium borohydride. We capped the tube with a rubber stopper and inserted a syringe needle. We incubated this solution at 37°C for 1 h and then cooled and neutralized it with glacial acetic acid. We purchased other folate standards of 5-methyltetrahydrofolate (5-mTHF) and FA from Sigma-Aldrich.

We purified all of the folate standards on a diethylaminoethyl cellulose column. We measured the purity and concentration of each standard separately with the use of a spectrophotometer (PerkinElmer LAMBDA 7 ultraviolet/Vis; Waltham, MA). We used the quantified standards to make external standards of 1 nmol/mL in 1% sodium ascorbate. We made fresh standards every month and stored them at −80°C in evacuated tubes.

We identified the folate forms by comparison of retention times and characteristic response across channels with those of the external standards. We ran the external standard mixtures that contained 20 pmol each of 5-mTHF, eTHF, and FA per mL at the start and end of each batch. To quantify 5-mTHF and FA in plasma, we added 20 pmol of eTHF to the sample and extraction buffer before extraction. We calculated the sum of the integrated areas of all peaks in channels that showed a response (in μA) for 5-mTHF and FA, multiplied the total by 20 pmol (the number of pmol of eTHF), and divided the result by the total area of all integrated peaks (in μA) for eTHF.

TABLE 1
Operating sequence for the chromatographic system to analyze plasma folate[1]

Time	Affinity column 1 (switching valve: position 1)	Time	Affinity column 2 (switching valve: position 1)
		min	
−4 to 0 min	Pump A: 0.5 mL/min for affinity column equilibration; sample loading into 900-μL loop	−4 to 3	Pump B: 1 mL/min of 70% A and 30% B for elution of folate into the analytic column, preparation for folate fractionation
>0 to 2 min	Injection signal and sample loading into the affinity column	0	Signal to start ESA recording, begin folate fractionation
>2 to 25 min	Washing of affinity column	>3 to 13	Pump B: 1 mL/min, 10% A and 90% B
>25 min	End of column wash and signal to valve to switch to position 2	>13 to 17	Pump B: 1 mL/min, 0% A and 100% B
		>17 to 25	Pump B: 1 mL/min 70% A and 30% B
		>25	End of folate analysis and signal to switch valve to position 2

[1] Pump A is the Waters pump (Milford, MA), which pumps 0.05 mmol/L potassium phosphate (pH 7) with 5% acetonitrile. Pump B is the Agilent pump (Santa Clara, CA), which pumps mobile phase A [28 mmol dibasic potassium phosphate/L and 60 mmol phosphoric acid/L (pH 3.0)] and B (the same as A with the addition of 20% acetonitrile). ESA, ESA CoulArray electrochemical detector (Chelmsford, MA).

Method linearity and sensitivity

We injected standards that contained 0.5–200 pmol per injection for 5-mTHF, eTHF, and FA in triplicate to show the linearity of signals. We calculated limits of detection for 5-mTHF and FA by serial dilution of a plasma sample with water (10–100 times) and quantification of each sample.

Recovery of standards

To measure the recovery of added folate standards to plasma, we extracted 10 aliquots of the same plasma sample. We ran 5 of the plasma extracts alone and added 20 pmol of 5-mTHF, FA, and eTHF to the other 5 plasma extracts before extraction. We also ran 5 standards of 20 pmol each of 5-mTHF, FA, and eTHF. We calculated the amount of folate from the spiked plasma minus the amount of folate and divided the results by the amount of folate from the standard alone to measure recovery.

Precision

We measured CVs for intra- and interassay precision for 5-mTHF and FA with the use of eTHF as an internal standard in plasma. We extracted a plasma sample and ran it in triplicate on the same day for intraassay precision. For interassay precision, we ran the plasma sample in triplicate on 3 separate days over 2 wk.

Microbial assay for folate

We measured total folate of the plasma samples by microbial assay with the use of *Lactobacillus casei*, as we have described previously (14, 15). Briefly, we serially diluted 5 μL of each plasma sample and plated the samples in triplicate onto a 96-microtiter well plate with 150 μL of *L. casei* growth medium. We incubated the plates overnight in a 37°C humid incubator and measured the absorbance, which indicated microbial growth, with the use of a 96-well plate reader (PowerWave HT; BioTek Instruments Inc, Winooski, VT) at 595 nm.

RESULTS

Chromatograms of folate forms

Examples of chromatograms for the external standards are shown in **Figure 2**A and a plasma sample with eTHF as the internal standard is shown in Figure 2B. The forms of folate we observed in plasma were 5-mTHF and FA. We also extracted folates from RBC samples and separated the polyglutamyl forms of methylfolate (Figure 2C).

Folate form distributions in plasma samples

We extracted folates from 168 archival fasted plasma samples. The median of 5-mTHF with the use of the HPLC method was 39.64 (range: 3.82–218.2) pmol/L. For unmetabolized FA concentrations, the median was 0.63 (range: 0–2.54) pmol/L. On average, unmetabolized FA accounted for 1.57% of total folate, with a range of 0–11.71%. We could detect unmetabolized FA in 43.4% of the samples. We detected no other forms of folate in these samples.

TABLE 2
Operating sequence for chromatography with the use of 2 affinity columns to analyze folate in red blood cells[1]

Time	Affinity column 1 (switching valve: position 1)	Time	Affinity column 2 (switching valve: position 1)
		min	
−4 to 0 min	Pump A: 0.35 mL/min for affinity column equilibration, sample loading into 900-μL loop	−4 to 3	Pump B: 1 mL/min of 90% A and 10% B for elution of folate into the analytic column, preparation for folate fractionation
>0 to 2 min	Injection signal and sample loading into the affinity column	0	Signal to start ESA recording, begin folate fractionation
>2 to 50 min	Washing of affinity column	0 to 5	Pump B: 1 mL/min, 90% A and 10% B
50.01 min	End of column wash and signal to valve to switch to position 2	>5 to 15	Pump B: 1 mL/min, 70% A and 30% B
		>15 to 40	Pump B: 1 mL/min 45% A and 55% B
		>40 to 50	Pump B: 1 mL/min 35% A and 65% B
		50.01	End of folate analysis and signal to valve to switch to position 2

[1] Pump A is the Waters pump (Milford, MA), which pumps 0.05 mmol/L potassium phosphate (pH 7) with 5% acetonitrile. Pump B is the Agilent pump (Santa Clara, CA), which pumps mobile phase A [28 mmol dibasic potassium phosphate/L and 60 mmol phosphoric acid/L (pH 3.0)] and B (same as A with the addition of 20% acetonitrile). ESA, ESA CoulArray electrochemical detector (Chelmsford, MA).

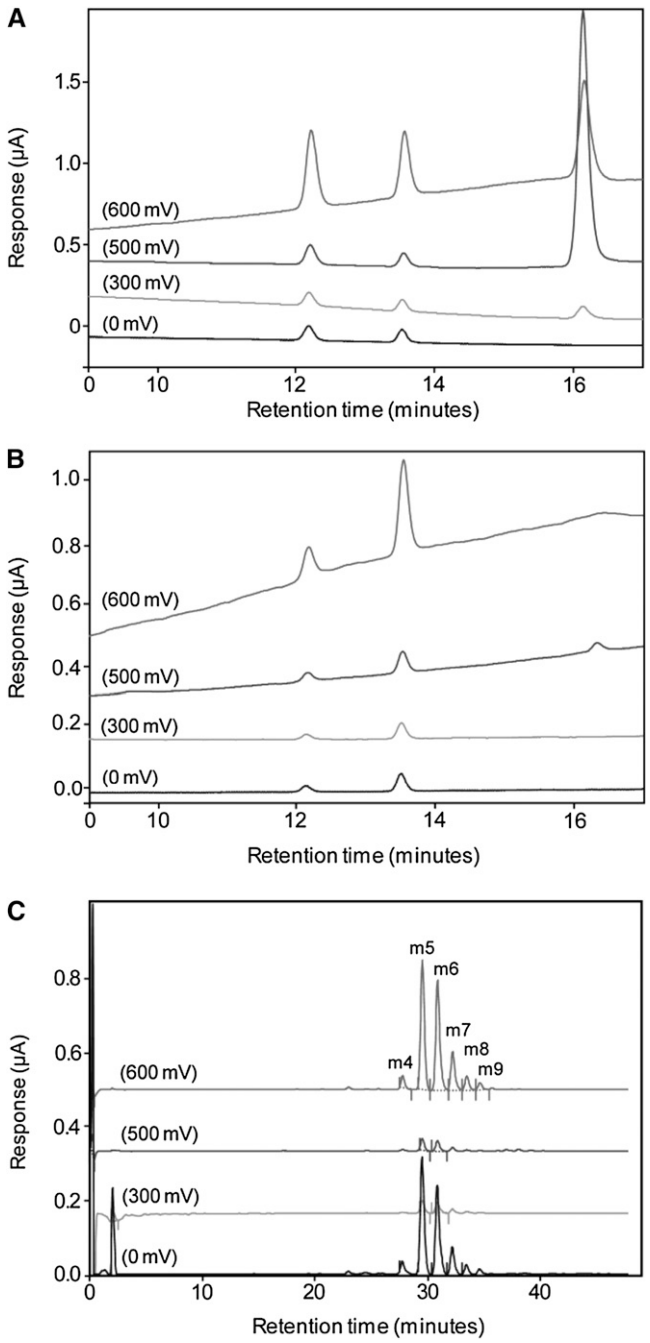

FIGURE 2. Chromatograms of external standards (A), human plasma sample (B), and red blood cells (C). The panels show the electrochemical response of channels 1, 2, 3, and 4 at 0, 300, 500, and 600 mV, respectively. A: Chromatogram for 20 pmol each of standards 5-methyltetrahydrofolate (M), ethyltetrahydrofolate (E), and folic acid (F). B: Chromatogram of a plasma sample that contained 5-methyltetrahydrofolate (M), ethyltetrahydrofolate (E), and folic acid (F). C: Chromatogram of red blood cell folate analysis. The peaks represent 5-methyltetrahydrofolate (m) with 4, 5, 6, 7, 8, and 9 glutamic acid residues.

Method validation

We showed a linear relation between the peak area and the concentration of each folate form over the ranges we tested (r^2 = 0.997–0.998). When we used a signal-to-noise ratio of 3, the limit of detection was 0.034 pmol for 5-mTHF and 0.027 pmol for FA per injection. These values corresponded to 0.26 pmol/mL plasma for 5-mTHF and 0.21 pmol/mL for FA. In a few samples, we detected FA even at concentrations below the calculated limit of detection. The recovery rate (mean ± SE) for samples spiked with standards was 92.2 ± 2.1% for 5-mTHF, 98.9 ± 5.2% for FA, and 93.8 ± 4.0% for eTHF. CVs for samples were 8.1% (within day) and 6.8% (between day) for 5-mTHF and 3.2% (within day) and 5.9% (between day) for FA (**Table 3**).

Comparison of HPLC and microbial assays for folate measurement

The correlation between the HPLC and *L. casei* methods was 0.979 ($P < 0.001$). On average, the HPLC yielded total folate values that were 1.09-fold higher (mean 1.09 ± 0.23 SD) than the *L. casei* method. The HPLC method typically produces values that are higher than those of the *L. casei* method (13, 16). The lower activity level we observed in the *L. casei* assay was probably a consequence of a small loss of folate in the microbiological assay during the required 24-h incubation period. The mean difference between the HPLC and *L. casei* assay results was 1.65 pmol/mL, which was not significant (95% CI for the mean difference: −0.053, 0.004).

DISCUSSION

In its original design, the affinity/HPLC system for assessment of folate form distribution is based on the use of purified and immobilized high-affinity folate binding protein on a matrix to concentrate and purify folate from biological samples. The next steps are elution and fractionation of retained folates by reverse-phase column chromatography and measurement of folate activity with the use of multichannel ultraviolet absorption that a diode array system facilitates. A single chromatography provides information on both the pteridine ring/one-carbon substitution structures and the extent of polyglutamation of individual folates (17).

We have used this method previously to assess folate form distribution in a number of animal tissues for different experimental models, and in a number of food products (18–20). Nevertheless, the original method has some disadvantages. The method uses sepharose as the matrix for the immobilized folate binding protein (21), which requires manual injection of the sample into the HPLC system. Another disadvantage is that ultraviolet detection is relatively insensitive and the system therefore requires a considerable amount of the biological specimen to allow detection of all activity peaks.

The modified affinity/HPLC with electrochemical detection method, which we also devised (13), used a polymer-based matrix for folate binding protein immobilization, which thus allowed online placement of the affinity column for automatic sample injection. The

TABLE 3
Performance statistics for the affinity HPLC method[1]

	Intraassay (n = 3)		Interassay (n = 9)	
Folate form	Mean	CV	Mean	CV
	ng/mL	*%*	*ng/mL*	*%*
5-mTHF	16.9	8.1	16.2	6.8
Folic acid	31.5	3.2	32.1	5.9

[1] To convert folate values to nanomoles per liter, multiply the amount by 2.266. CV was calculated with the use of Microsoft Excel software (Seattle, WA). 5-mTHF, 5-methyltetrahydrofolate.

2-position 6-port Rheodyne valve between the affinity column and the analytic column allowed separation of the 2 columns during loading and subsequent washing of the affinity column. The use of a 4-channel electrochemical detector from ESA set at different voltages allowed identification of the different folate forms at a 10–20-fold higher sensitivity than with ultraviolet detection. Modifications also included a different analytic column (Betasil C18; Keystone), which served as an immobilized ion pair, and a different mobile phase. The ion pairing mobile phase system that the old method used is not feasible with electrochemical detection.

We have used this affinity/HPLC with electrochemical detection method to show the presence of nonmethylated THF polyglutamates in RBC from homozygotes for the 677T mutation in the *MTHFR* gene (22). We have also used the method to show the high concentrations of unmetabolized FA and 5-mTHF in patients undergoing kidney dialysis who were receiving high doses of FA (23). Finally, we have used the method in a study that showed an inverse correlation between unmetabolized FA (but not 5-mTHF) in plasma and natural killer cell activity (10).

Even with the system's many improvements, analysis of a single sample took 75 min, which makes this method impractical for epidemiologic analyses. In this article, we describe how we modified this method to measure unmetabolized FA in plasma for epidemiologic studies. Instead of one affinity column, we used 2 columns that we linked to a 2-position, 10-port switching valve. This approach facilitated the ability to load and subsequently wash one column while the other column was eluted into the analytic column for folate fractionation and subsequent electrochemical analysis (Table 1). Except for the first cycle, which took 60 min to generate the first set of data, subsequent cycles provided data every 30 min, and we could run ≈45–47 samples every 24 h.

Another improvement is the use of synthetic eTHF as an internal standard to account for any loss of folate during extraction and subsequent binding to the affinity column. The usefulness of eTHF as an internal standard is evident from the low intra- and interassay CVs and high correlations between the total folate (ie, FA plus 5-mTHF) that we had determined by this method and the *L. casei* assay.

In summary, this method is suitable for population studies because of its high throughput, requirement for only a small amount of plasma, and use of an internal standard. Researchers can also use this method to analyze folate form distribution in cells. Further research is needed to determine whether measurement of unmetabolized FA in plasma samples would provide additional information to recent suggestions that fortification with FA has an association with adverse health outcomes (10–12).

The authors' responsibilities were as follows—JS: designed the research; RK, LP, and JS: conducted the research and wrote the manuscript; RK: analyzed the data; and JS: had primary responsibility for the final content. The authors had no conflicts of interest.

REFERENCES

1. U.S. Food and Drug Administration. Food standards: amendment of standards of identity for enriched cereal grain products to require addition of folic acid. Final Rule. Federal Register 1996;61:8781–97.
2. Eichholzer M, Tonz O, Zimmermann R. Folic acid: a public-health challenge. Lancet 2006;367:1352–61.
3. Yang Q, Botto LD, Erickson JD, et al. Improvement in stroke mortality in Canada and the United States, 1990 to 2002. Circulation 2006;113:1335–43.
4. Jacques PF, Selhub J, Bostom AG, Wilson PW, Rosenberg IH. The effect of folic acid fortification on plasma folate and total homocysteine concentrations. N Engl J Med 1999;340:1449–54.
5. Choumenkovitch SF, Jacques PF, Nadeau MR, Wilson PW, Rosenberg IH, Selhub J. Folic acid fortification increases red blood cell folate concentrations in the Framingham study. J Nutr 2001;131:3277–80.
6. Lewis CJ, Crane NT, Wilson DB, Yetley EA. Estimated folate intakes: data updated to reflect food fortification, increased bioavailability, and dietary supplement use. Am J Clin Nutr 1999;70:198–207.
7. Pfeiffer CM, Caudill SP, Gunter EW, Osterloh J, Sampson EJ. Biochemical indicators of B vitamin status in the US population after folic acid fortification: results from the National Health and Nutrition Examination Survey 1999-2000. Am J Clin Nutr 2005;82:442–50.
8. Kelly P, McPartlin J, Goggins M, Weir DG, Scott JM. Unmetabolized folic acid in serum: acute studies in subjects consuming fortified food and supplements. Am J Clin Nutr 1997;65:1790–5.
9. Sweeney MR, McPartlin J, Weir DG, Daly L, Scott JM. Postprandial serum folic acid response to multiple doses of folic acid in fortified bread. Br J Nutr 2006;95:145–51.
10. Troen AM, Mitchell B, Sorensen B, et al. Unmetabolized folic acid in plasma is associated with reduced natural killer cell cytotoxicity among postmenopausal women. J Nutr 2006;136:189–94.
11. Selhub J, Morris MS, Jacques PF. In vitamin B12 deficiency, higher serum folate is associated with increased total homocyteine and methylmalonic acid concentrations. Proc Natl Acad Sci USA 2007;104:19995–20000.
12. Wright AJ, Dainty JR, Finglas PM. Folic acid metabolism in human subjects revisited: potential implications for proposed mandatory folic acid fortification in the UK. Br J Nutr 2007;98:667–75.
13. Bagley PJ, Selhub J. Analysis of folate form distribution by affinity followed by reversed- phase chromatography with electrical detection. Clin Chem 2000;46:404–11.
14. Horne DW, Patterson D. Lactobacillus casei microbiological assay of folic acid derivatives in 96-well microtiter plates. Clin Chem 1988;34:2357–9.
15. Tamura T, Freeberg LE, Cornwell PE. Inhibition of EDTA of growth of Lactobacillus casei in the folate microbiological assay and its reversal by added manganese or iron. Clin Chem 1990;36:1993.
16. Poo-Prieto R, Haytowitz DB, Holden JM, et al. Use of the affinity/HPLC method for quantitative estimation of folic acid in enriched cereal-grain products. J Nutr 2006;136:3079–83.
17. Selhub J. Determination of tissue folate composition by affinity chromatography followed by high-pressure ion pair liquid chromatography. Anal Biochem 1989;182:84–93.
18. Hidiroglou N, Camilo ME, Beckenhauer HC, et al. Effect of chronic alcohol ingestion on hepatic folate distribution in the rat. Biochem Pharmacol 1994;47:1561–6.
19. Selhub J, Seyoum E, Pomfret EA, Zeisel SH. Effects of choline deficiency and methotrexate treatment upon liver folate content and distribution. Cancer Res 1991;51:16–21.
20. Varela-Moreiras G, Selhub J. Long-term folate deficiency alters folate content and distribution differentially in rat tissues. J Nutr 1992;122:986–91.
21. Selhub J, Ahmad O, Rosenberg IH. Preparation and use of affinity columns with bovine milk folate-binding protein (FBP) covalently linked to Sepharose 4B. Methods Enzymol 1980;66:686–90.
22. Bagley PJ, Selhub J. A common mutation in the methylenetetrahydrofolate reductase gene is associated with an accumulation of formylated tetrahydrofolates in red blood cells. Proc Natl Acad Sci USA 1998;95:13217–20.
23. Ghandour H, Bagley PJ, Shemin D, et al. Distribution of plasma folate forms in hemodialysis patients receiving high daily doses of L-folinic or folic acid. Kidney Int 2002;62:2246–9.