B Vitamins and Related Biochemical Compounds

Sources and Physiological Functions

Folate, vitamin B6, and vitamin B12 belong to the group of water-soluble B vitamins that occur naturally in food (Institute of Medicine 1998). Leafy green vegetables (such as spinach and turnip greens), fruits (such as citrus fruits and juices), and dried beans and peas are all natural sources of folate. Folic acid is a folate vitamer found in supplements and fortified foods. Because of wide consumption of fortified foods in the United States, these products have become an important contributor of folic acid to the U.S. diet (Zhou 2023). Folate functions as a coenzyme in one-carbon transfers in the metabolism of nucleic and amino acids. It is therefore especially important during periods of rapid cell division and growth, such as occurs during pregnancy and infancy (Bailey 2015). The term "folate" represents the sum of different folate vitamer compounds.

The most abundant dietary sources of vitamin B6 are meats, whole grains, vegetables, and nuts. Vitamin B6 is used as a cofactor for over 100 biochemical reactions in the human body related to amino acid metabolism, one-carbon metabolism, glycogenolysis, and gluconeogenesis (Institute of Medicine 1998). The three major vitamin B6 forms are pyridoxine (the major form in plants), pyridoxal, and pyridoxamine (these two are the most abundant forms in humans and animals). Pyridoxal-5'-phosphate (PLP) is the most biologically active coenzyme form. 4-Pyridoxic acid (4PA) is the end product of vitamin B6 catabolism (Institute of Medicine 1998).

Vitamin B12 (cobalamin) is found naturally in animal foods, including fish, meat, poultry, eggs, milk, and milk products. Vegetarians and vegans may rely on fortified foods as their primary vitamin B12 source (Pawlak 2014). Vitamin B12 functions as a coenzyme for two critical methyl transfer reactions that convert homocysteine to methionine and L-methylmalonyl-coenzyme A to succinyl-coenzyme A (Allen 2018).

Homocysteine (HCY) is an amino acid naturally found in the blood. Plasma HCY concentrations are strongly influenced by diet and genetic factors (Refsum 2004). Elevated concentrations of total HCY (tHCY, the sum of free, protein-bound, and disulfides) are found in people whose folate, vitamin B12, and/or vitamin B6 status is suboptimal (Selhub 1993) and in people with impaired renal function (Wollensen 1999).

Methylmalonic acid (MMA) is a dicarboxylic acid naturally found in the blood. Plasma MMA concentrations are elevated when serum vitamin B12 concentrations are low or intermediate and

are therefore a useful diagnostic test for confirming vitamin B12 deficiency (Hølleland 1999; Baik 1999). As with plasma tHCY, MMA concentrations are also elevated in people with impaired renal function (Rasmussen 1990).

Health Effects

Isolated dietary deficiencies of either folate, vitamin B6, or vitamin B12 are rare in the United States (Pfeiffer 2019; Morris 2008; Mineva 2021). Folate and vitamin B6 deficiencies usually coexist with other nutrient deficiencies because of their strong association with poor diet, alcoholism, or malabsorptive disorders (Carmel 2005; McCormick 2006). Most people who develop vitamin B12 deficiency have an underlying stomach or intestinal disorder that limits the absorption of vitamin B12 (Institute of Medicine 1998).

The primary clinical sign of folate or vitamin B12 deficiency is megaloblastic anemia, while vitamin B6 deficiency is associated with microcytic anemia (Institute of Medicine 1998). Less specific signs of deficiency for these B vitamins can include weakness, fatigue, heart palpitations, confusion, depression, dermatitis, and glossitis (inflammation of the tongue) (Institute of Medicine 1998). Severe vitamin B12 deficiency can cause permanent nerve damage and dementia (Johnson 2024). Hematologic signs, however, are not always present in vitamin B12 deficiency, and hematologic signs and neurologic abnormalities can be inversely correlated (Baik 1999).

Certain drugs (e.g., alcohol, methotrexate, anticonvulsants, sulfa drugs) may interfere with the absorption or utilization of folate (Institute of Medicine 1998). Disorders of the small bowel that limit absorption (e.g., Crohn's disease, jejunal bypass surgery) can cause folate deficiency (Halsted 1990). Drugs that react with carbonyl groups have the potential to interact with PLP (Institute of Medicine 1998). Isoniazid—used in the treatment of tuberculosis—and L-DOPA have been shown to reduce plasma PLP concentrations (Institute of Medicine 1998). A small decrease in vitamin B6 status has been seen in women taking high-dose oral contraceptives (Institute of Medicine 1998). An inverse association between plasma PLP and elevated plasma C-reactive protein (CRP) was shown to be independent of vitamin B6 intake (Morris 2010) and of plasma tHCY (Friso 2001). The inverse association suggests that inflammation is associated with a functional deficiency of vitamin B6 (Sakakeeny 2012). Vitamin B6 turnover is increased during inflammation, as evidenced by a higher ratio of 4PA/PLP, and was also found to be associated with greater mortality risk in the general U.S. population (Schorgg 2022).

Clinical trials have shown that folic acid supplementation effectively reduces the number of neural tube birth defects (NTDs) (Berry 1999; Czeizel 1992; MRC Vitamin Study Research Group 1991). In 1992, the U.S. Public Health Service recommended that every woman who could become pregnant consume at least 400 micrograms (µg) of folic acid each day (U.S. Centers for Disease Control and Prevention 1992). The CDC recommendation was reinforced by the U.S. Preventive Services Task Force in 2009 (U.S. Preventive Services Task Force 2009) and reaffirmed as a Grade A recommendation in 2023 (U.S. Preventive Services Task Force 2023). Since 1998, the U.S. Food and Drug Administration (FDA) has required the addition of folic acid to enriched breads, cereals, flours, corn meals, pastas, rice, and other grain products (U.S. Food and Drug Administration 1996). After the introduction of fortification, NTD rates in the United States have decreased by 19%–32% (Crider 2011). Nevertheless, rates were still highest among Hispanic women (Boulet 2008), possibly due to their lower consumption of total folic acid, which is specifically true for less acculturated populations (Hamner 2011). In 2016, the FDA approved the voluntary addition of folic acid to corn masa flour, a staple food for many Latin Americans in the United States (U.S. Food and Drug Administration 2016). Voluntary folic acid fortification has not produced a meaningful change in RBC folate status or in model-based NTD rates in Hispanic women of reproductive age (Wang 2021; Wang 2024), possibly because corn masa flour fortification was not adopted by manufacturers (Redpath 2018). Factors beyond folate status (e.g., genetic, environmental) that modulate NTD prevalence could contribute to the remaining risk for folate-sensitive NTDs.

Observational studies have suggested potential benefits of folic acid fortification beyond the reduction of NTD rates. These benefits include decreased prevalence of folate deficiency based on serum and RBC folate concentrations (Pfeiffer 2007; Pfeiffer 2019) and declines in the incidence of stroke (Yang 2006) and neuroblastoma (French 2003).

Research continues on the potential roles of B vitamins in modulating the risk for diseases such as cardiovascular disease (CVD), cancer, and cognitive impairment. The link between moderately elevated plasma tHCY levels and chronic conditions was based on observational studies with mixed results. Short-term supplementation trials with mixed folic acid, vitamin B12, or vitamin B6 for secondary prevention of CVD have yielded largely negative results (Clarke 2011). A meta-analysis on the HCY lowering of B vitamins showed no significant effect on individual cognitive domains, global cognitive function, or cognitive aging (Clarke 2014). There is controversy, however, about whether plasma tHCY is a modifiable risk factor for the development of cognitive decline,

dementia, and Alzheimer's disease in older persons (Smith 2018). An umbrella review of meta-analyses and Mendelian randomization studies on HCY and multiple health outcomes found convincing evidence that HCY exposure plays a clear—but likely not causal—role in digestive tract cancer and likely a causal role in stroke based on intervention trials (Zhou 2025).

Several observational studies pointed to a link between low B vitamin status and negative health outcomes, albeit with mixed results. A meta-analysis of prospective cohort studies revealed a significant inverse relation between dietary folate intake and risk of all-cause and CVD mortality but not with folate biomarkers (Fallah 2025). A study of the association of one-carbon metabolism-related compounds with epigenetic aging biomarkers in a nationally representative sample of the United States found that higher folate concentrations, a carbon donor, were associated with lower epigenetic age deviation, while higher tHCY concentrations, an indicator of one-carbon metabolism deficiencies, were associated with greater epigenetic age deviation, with some associations influenced by smoking and renal function (Bozack 2025).

Increasing the proportion of women of childbearing age who get enough folic acid is one of the objectives for Healthy People 2030. Part of this objective is also to increase the proportion of women of childbearing age who have optimal red blood cell folate concentrations. To provide data for this objective, the National Health and Nutrition Examination Survey (NHANES) continues to monitor folate status in the U.S. population.

Intake Recommendations

The recommended dietary allowance (RDA) for both men and women is 400 μg per day of dietary folate equivalents (DFEs). DFEs adjust for the nearly 50% lower bioavailability of dietary folate compared to the bioavailability of folic acid. 1 μg of dietary folate equivalent equals 0.6 μg of folic acid from fortified food or from a supplement taken on an empty stomach (Institute of Medicine 1998). The RDA for vitamin B6 is 1.3 mg for men and women ages 19–50 years, 1.7 mg for men and 1.5 mg for women ages 51 years and older, and 1.9 mg for pregnant women (2.0 mg if lactating) (Institute of Medicine 1998). The RDA for vitamin B12 for adults is 2.4 μg per day. Because as many as 10% to 30% of older people may be unable to absorb naturally occurring vitamin B12, people older than age 50 years should meet their RDA mainly by consuming foods fortified with vitamin B12 or by taking a supplement containing vitamin B12. People with vitamin B12 deficiency caused by a lack of intrinsic factor or intestinal malabsorption require parenteral B12 treatment (Institute of Medicine 1998).

Prolonged consumption of very high daily intakes of folic acid has the potential to delay the diagnosis of anemia among adults with vitamin B12 deficiency. This may result in increased risk of progressive, unrecognized neurological damage from untreated vitamin B12 deficiency. However, epidemiological evidence suggests little risk of folic acid fortification masking vitamin B12 deficiency (Crider 2022). The Institute of Medicine (1998) set the Tolerable Upper Intake Level (UL) for folic acid intake for adults (ages 19 years and older) at 1000 µg per day. The UL is defined as the "maximum daily intake levels at which no risk of adverse health effects is expected for almost all individuals in the general population—including sensitive individuals—when the nutrient is consumed over long periods of time" (Institute of Medicine 2000). Because no data were available for children, the Institute of Medicine used the UL for adults adjusted by weight: 300–800 µg per day, depending on the age group. Folate intake from food is not associated with any health risk. The UL for vitamin B6 for adults is 100 mg per day (Institute of Medicine 1998). If more is ingested through supplements, sensory neuropathy, dermatological lesions, and reversible nerve damage to the arms and legs can occur (Institute of Medicine 1998). No adverse effects have been seen, however, from getting large amounts of vitamin B6 through food sources (Institute of Medicine 1998). No adverse effects have been associated with excess vitamin B12 intake from food or supplements in healthy individuals, and no UL has been set (Institute of Medicine 1998).

Two authoritative bodies, the U.S. National Toxicology Program and the UK Scientific Advisory Committee on Nutrition, have convened expert panels to assess risks from high intakes of folic acid (Field 2018). The totality of the evidence has not established risks for adverse consequences resulting from existing mandatory folic acid fortification programs that have been implemented in many countries (National Toxicology Program 2015; Public Health England 2017; Maruvada 2020). However, additional research is needed to assess the health effects of folic acid supplement use when the current upper limit for folic acid is exceeded.

Biochemical Indicators and Cutoff Values

Folate. The Biomarkers of Nutrition for Development (BOND) project published a comprehensive review covering relevant aspects of folate biology and biomarkers (Bailey 2015). Folate status can be assessed by measuring serum or plasma "total" folate, which provides information on recent intake, and RBC "total" folate, which is indicative of body folate stores and long-term nutritional status. "Total" folate is composed of different folate vitamers, such as 5-methyltetrahydrofolate (5-methylTHF) and folic acid. Serum 5-methylTHF is the main circulating form and contributes >80% to "total" folate. Folic acid (4%), non-methyl folate (4.7%), and MeFox (4.5%), a 5-methylTHF oxidation product (pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF), contribute smaller amounts (Pfeiffer 2015a). Because the human gut has a limited ability to reduce and methylate folic acid derived from supplements and fortified foods (Patanwala 2014), folic acid can appear in circulation. Other folate vitamers such as tetrahydrofolate and formyl-folates may also appear in circulation, though generally only at trace concentrations (Pfeiffer 2015a). RBCs contain mainly 5-methylTHF polyglutamates as a storage form. However, the 5,10-methenyltetrahydrofolate reductase (*MTHFR*) C677T polymorphism introduces a bottleneck in folate metabolism, a so-called "methyl-trap", which impacts the conversion of cellular formyl-folate to methyl-folate, leading to notable non-methyl accumulation in red blood cells (Smulders 2007).

Different cutoff values have been used to assess various degrees of folate sufficiency by different assays (WHO 2015a). Originally, cutoff values indicative of deficiency were based on concentrations at which megaloblastic anemia is likely to appear. They were derived with traditional microbiologic assays (MBA-1; wild-type microorganism and calibrated with folic acid): <3 ng/mL (6.8 nmol/L) for serum folate and <100 ng/mL (227 nmol/L) for RBC folate (WHO 1968). A higher cutoff of 140 ng/mL (305 nmol/L) is more typical for RBC folate (Institute of Medicine 1998). Studies from Ireland (Daly 1995) and China (Crider 2014) showed a consistent relationship between increasing risk of NTD-affected pregnancies and decreasing RBC folate concentrations.

These studies contributed to a 2015 WHO guideline on optimal serum and RBC folate concentrations in women of reproductive age for prevention of folate-sensitive NTDs (WHO 2015b). The guideline contained four recommendations:

- At the population level, RBC folate concentrations should be >400 ng/mL (906 nmol/L) in women of reproductive age to achieve the greatest reduction of NTDs.
- The RBC folate threshold can be used as an indicator of folate insufficiency in women of reproductive age.
- No serum folate threshold is recommended for prevention of NTDs in women of reproductive age at the population level.
- The microbiologic assay is the most reliable choice of obtaining comparable results for RBC folate concentrations across countries.

The RBC folate cutoff value of 906 nmol/L for folate insufficiency (MBA-2; chloramphenicol-resistant microorganism and calibrated with folic acid) corresponds to a cutoff of 748 nmol/L (MBA-3; chloramphenicol-resistant microorganism and calibrated with 5-methylTHF; CDC microbiologic assay used in NHANES from 2007 onward) (Pfeiffer 2016). A 2005 WHO Technical Consultation on folate deficiency estimated blood folate concentrations below which tHCY, a functional indicator of folate deficiency, showed elevated concentrations: 4 ng/mL (10 nmol/L) for serum folate and 151 ng/mL (340 nmol/L) for RBC folate (de Benoist 2008). These cutoff values were derived from NHANES III (1988–1994) data generated with the BioRad radioprotein-binding assay, which measured lower than the CDC microbiologic assay (Pfeiffer 2012a). When adjusted to the MBA-3, these cutoff values were equivalent to 14 nmol/L for serum folate and 624 nmol/L for RBC folate (Pfeiffer 2016). These cutoffs should be used with caution as they only apply to the MBA-3 assay. Furthermore, they were derived from cross-sectional pre-fortification NHANES data and a validation of these cutoffs through experimental data is lacking (Pfeiffer 2016).

Vitamin B6. Vitamin B6 status is typically assessed by measuring the level of one or more B6 vitamers in serum or plasma. Serum PLP is generally viewed as the best single indicator of status. Serum or urinary 4PA, the end product of vitamin B6 catabolism, is an indicator of recent intake. The Institute of Medicine has established a PLP cutoff value of 20 nmol/L as the basis for the Estimated Average Requirement (EAR) (Institute of Medicine 1998). This cutoff may overestimate the vitamin B6 requirement for health maintenance of more than half the group. Serum PLP

concentrations in the range of 20–30 nmol/L are often described as marginal vitamin B6 status (da Silva 2020).

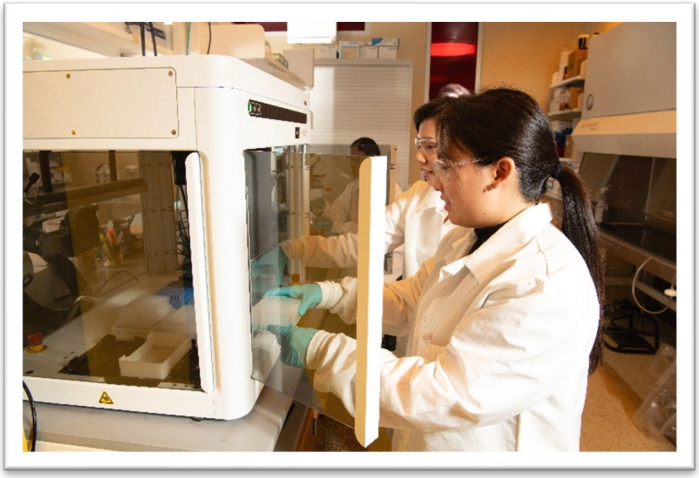
Vitamin B12. Vitamin B12 status can be assessed by measuring serum or plasma total cobalamins or serum holo-transcobalamin II, the transport protein of absorbed cobalamin. Urine or serum MMA is a specific functional indicator of vitamin B12 status. Plasma tHCY is a functional indicator of folate, vitamin B6, and/or B12 status, but it is not specific for any of these vitamins. As B vitamin concentrations decrease, plasma tHCY concentrations increase; as serum vitamin B12 concentrations decrease, plasma MMA concentrations increase. A widely used cutoff value for serum vitamin B12 in the clinical setting is 200 pg/mL (148 pmol/L). Higher cutoff values (300 pg/mL [222 pmol/L] or 350 pg/mL [258 pmol/L]) are also sometimes used with increasing sensitivity but decreasing specificity (Carmel 2011). The WHO consensus cutoff value of 203 pg/mL (150 pmol/L) estimates serum vitamin B12 concentrations below which plasma MMA concentrations become elevated (de Benoist 2008). No consensus exists on what serum vitamin B12 cutoff value(s) should be used in population-based research to define vitamin B12 status (Bailey 2011). Modeling of an MMA-derived cutoff value for serum vitamin B12 in adults in NHANES suggested the existence of two cutoff values (<126 pmol/L and >287 pmol/L). The vitamin B12 status of the intermediate group (33% of adults) is difficult to interpret (Bailey 2013).

tHCY and MMA. A frequently used cutoff value for elevated plasma tHCY concentrations is 15 µmol/L (NACB 2009). Commonly used cutoff values for elevated plasma MMA concentrations are 271 nmol/L and 376 nmol/L (Allen 1990). The higher MMA cutoff value provides more specificity, particularly when MMA is confounded by impaired renal function. Age-specific MMA reference intervals may be appropriate, given that older persons who are vitamin B12-replete and have normal renal function have higher serum MMA concentrations than their younger counterparts (Vogiatzoglou 2009; Mineva 2019).

Combined indicator of vitamin B12 status (cB12). The combined indicator of vitamin B12 status (cB12) is an algorithm-based biomarker. It integrates measurements of up to four vitamin B12 status biomarkers (i.e., serum B12, MMA, tHCY, and holotranscobalamin (holoTC)) while adjusting for the effects of age and folate status (Fedosov 2010; Fedosov 2013; Fedosov 2015). By combining multiple indicators, cB12 helps to improve the sensitivity and specificity of diagnosing vitamin B12 deficiency (Fedosov 2010). This could be particularly beneficial in cases where individual markers may be affected by factors such as renal function or folate status. Proposed epidemiological

cutoffs for cB12 to define vitamin B12 status are as follows: cB12 <-2.5 (low), cB12 between -0.5 and -2.5 (transitional), and cB12 >-0.5 (adequate) (Fedosov 2013). This indicator has limited established clinical utility and is primarily used for epidemiological research.

Analytical Methods



Folate. Because of observed method differences in measuring folate concentrations (Gunter 1996; Pfeiffer 2010), caution should be used in comparing values from different datasets and in using the above cutoff values (Pfeiffer 2016; Rogers 2018). Method-specific cutoff values and reference intervals for use in clinical diagnostics have been suggested (Life Sciences Research Office 1994; Gunter 1996) and may be required until clinical assays have been fully standardized (Pfeiffer 2016). The three main approaches to measure folate are via microbiologic assay (MBA), protein-binding assay, and chromatographic assay. Owing to its low cost and simple instrumentation, the MBA has experienced a resurgence, particularly for population status assessment in low-resource settings (Pfeiffer 2018). Harmonizing the calibrator and microorganism for the MBA has also led to improved comparability of serum and whole blood folate results (Zhang 2018). In clinical settings, commercial protein-binding assays on automated clinical analyzers are offering high throughput (Shane 2011). These assays may measure lower (Fazili 2007; Fazili 2008) or higher (Colapinto 2014) than the MBA. Relative to the microbiologic assay, they seem to respond differently to methyl-folate versus formyl-folate and thus may not correctly determine folate status depending on the *MTHFR* C677T polymorphism (Fazili 2008; Tsang 2015). This complicates the interpretation of data from different methods. The BioRad radioassay measured approximately 35% lower than the traditional MBA-1 (Life Sciences Research Office 1994). When compared to the CDC MBA-3, the BioRad assay measured on average 29% lower in serum samples and 45% lower in whole blood samples (Pfeiffer 2012a). In research settings, chromatography-based methods, nowadays coupled to tandem mass spectrometry (HPLC-MS/MS), are often used to measure individual forms of folate in serum or whole blood (Pfeiffer 2010). In an international round robin study, CDC compared the performance

of different HPLC-MS/MS methods measuring serum folate forms (Fazili 2017). Good agreement was obtained for the measurement of 5-methylTHF, but laboratory performance for folic acid was highly variable and needed improvement.

A few international reference materials for serum folate from the U.S. National Institute of Standards and Technology (NIST) and the United Kingdom National Institute for Biological Standards and Control (NIBSC), with certified or reference values by higher-order reference methods (HPLC-MS/MS) for some folate vitamers have been developed: NIST SRM 1955 (Homocysteine and folate in frozen human serum; no longer available), NIST SRM 1950 (Metabolites in human plasma), NIST SRM 3949 (Folate vitamers in frozen human serum), and NIBSC 03/178 (Human serum vitamin B12 and folate). A reference material for whole blood folate is available from the NIBSC (95/528 Whole blood folate); however, the value assignment for this material was by consensus of protein-binding and MBAs. No international reference materials are available with certified "total" folate values, which complicates standardization efforts particularly for clinical assays measuring total folate.

Vitamin B6. Vitamin B6 forms in serum are most commonly measured by high performance liquid chromatography (HPLC) with fluorometric detection. Chemical derivatization (sample, online, or post-column) is almost always used to enhance PLP fluorescence (Rybak 2004). Enzymatic and microbiologic methods (Coburn 2000) and, more recently, HPLC-MS/MS methods have also been employed (Midttun 2005; Maus 2023). A CDC round robin study showed room for improvement in the comparability of HPLC and enzymatic methods (Rybak 2005). Since 2011, two international reference materials have provided certified concentrations for serum PLP by HPLC-MS/MS: NIST SRM 1950 (Metabolites in human plasma) and 3950 (Vitamin B6 in frozen human serum).

Vitamin B12, tHCY, and MMA. Serum vitamin B12 is commonly measured by competitive protein-binding assay (Carmel 2011). Research methods for tHCY determination are based on HPLC with fluorescence detection or coupled to tandem mass spectrometry; clinical methods are based on immunoassay or enzymatic principle (Refsum 2004). MMA

is measured by gas chromatography coupled to mass spectrometry (GC-MS) or by HPLC-MS/MS (Pedersen 2011; Mineva 2015). The comparability of methods for plasma tHCY (Pfeiffer 1999a) and MMA (Pfeiffer 1999b) is generally good, but questions have been raised about the comparability of serum vitamin B12 methods (Ispir 2015; Willis 2011). The following international reference materials are available: NIBSC 03/178 for serum vitamin B12 (consensus value) and NIST SRM 1950 for plasma tHCY (certified concentration by GC-MS).

Clinical laboratories typically use conventional units for measuring concentrations of folate (nanograms per milliliter [ng/mL]) and vitamin B12 (picograms [pg]/mL). They use international system (SI) units for vitamin B6 (nanomole per liter [nmol/L]), tHCY (micromole per liter [µmol]/L), and MMA (nmol/L). Conversion factors from conventional to SI units are as follows: 1 ng/mL = 2.266 nmol/L for folate and 1 pg/mL = 0.738 picomol (pmol)/L for vitamin B12.

Findings from NHANES

Folate. The National Health and Nutrition Examination Survey (NHANES) is the only source for nationally representative data on folate and other B vitamin biomarkers for the U.S. population (Pfeiffer 2026a). Monitoring the folate status of the U.S. population over time has been a priority for several decades (Yetley 2011). Earlier studies from NHANES II (1976–1980) (Life Sciences Research Office 1984; Senti 1985) and NHANES III (1988–1994) (Wright 1998) suggested that the folate status of some population groups might be of public health concern. After the introduction of folic acid fortification in 1998, serum and RBC folate concentrations increased substantially in all age groups (Pfeiffer 2007). During the first eight years of the continuous NHANES (1999–2006), folate data were generated by the commercial BioRad Quantaphase II radioprotein-binding assay kit (Yetley 2011), the same method as used during NHANES III (1988–1994). During NHANES 2007–2010, serum and RBC "total" folate were measured using an MBA (Pfeiffer 2011). Additionally, an HPLC-MS/MS method was used to measure serum 5-methylTHF and folic acid in a one-third subsample of NHANES 2007–2008 (Pfeiffer 2015b). To allow interpretation of folate status between 1988 and 2010, the earlier BioRad data were adjusted to MBA-equivalent data (Pfeiffer 2012a; Pfeiffer 2012b). Post-fortification serum and RBC folate concentrations were 2.5 times and 1.5 times pre-fortification concentrations, respectively, and during 1999–2010 concentrations declined slightly (Pfeiffer 2012b).

Folate status in the U.S. population was stable nearly 20 years after the introduction of folic acid fortification (Pfeiffer 2019). The prevalence for risk of folate deficiency based on megaloblastic

anemia as a hematologic indicator was 5.6% (serum folate) and 7.4% (RBC folate) pre-fortification (1988–1994). The prevalence was <1% (serum and RBC folate) post-fortification (1999–2010) (Pfeiffer 2016). Using post-fortification data from NHANES 2007–March 2020, 19.5% of nonpregnant women ages 12–49 years were characterized as folate insufficient (Wang 2025). The percentage was based on suboptimal RBC folate concentrations associated with increased NTD risk (<748 nmol/L, which is equivalent to the WHO cutoff value of 906 nmol/L after adjusting for assay differences) (Tinker 2015).

Serum folic acid was detected at low concentrations (median of 0.7 nmol/L in fasting persons) in nearly all participants in NHANES 2007–2008. Concentrations exceeding 1 nmol/L were largely but not entirely explained by fasting status and by total folic acid intake from diet and supplements (Pfeiffer 2015b). Some patterns observed with MeFox in NHANES 2011–2016 were different from those observed with 5-methylTHF (Pfeiffer 2015a; Fazili 2020). The differing patterns suggest that altered folate metabolism is dependent on biological characteristics. MeFox concentrations increased with age and showed no difference by sex, while 5-methylTHF concentrations showed a U-shaped age pattern and were higher in females (Pfeiffer 2015a; Fazili 2020). MeFox concentrations were much higher in persons with poor kidney function and those with higher BMI, while 5-methylTHF concentrations were only slightly higher in those with poor kidney function and were lower in those with higher BMI (Pfeiffer 2015a; Fazili 2020; Wang 2022). MeFox concentrations were also higher under conditions of uncontrolled diabetes (Crider 2026). After adjusting for demographic changes over time, mean concentrations of serum folate (~25%–30%) and RBC folate (~10%) decreased from NHANES 1999–2000 to August 2021–August 2023 (Pfeiffer 2026b) and the prevalence of folate insufficiency (RBC folate <748 nmol/L) increased from ~16% to 21% (Pfeiffer 2026c).

Vitamin B6. The vitamin B6 status of the U.S. population was monitored during NHANES 2003–2010, but two different methods that are not directly comparable have been used (U.S. Centers for Disease Control and Prevention 2010). In 2003–2004, serum PLP was measured using an enzymatic assay, while in 2005–2010 serum PLP and 4PA were measured using HPLC with post-column derivatization and fluorometric detection (Rybak 2004; Rybak 2009). Based on NHANES 2003–2004 data, substantial proportions of some population subgroups (smokers, the elderly, non-Hispanic blacks, and current and former oral contraceptive users) had plasma PLP concentrations <20 nmol/L. These people may not meet accepted criteria for adequate vitamin B6 status even

though they had vitamin B6 intakes consistent with the definition of a RDA (3 to 4.9 mg/d) (Morris 2008). In NHANES 2005–2006, the estimated prevalence of vitamin B6 deficiency (serum PLP <20 nmol/L) was 10.6% (U.S. Centers for Disease Control and Prevention 2012). Between NHANES 2005–2006 and 2009–2010, the prevalence of vitamin B6 deficiency and insufficiency (serum PLP <30 nmol/L) was relatively stable at ~10% and 25%, respectively (Pfeiffer 2026c).

Vitamin B12. The vitamin B12 status of the U.S. population was monitored during the second phase of NHANES III (1991–1994) (Wright 1998). The first eight years of the continuous survey (1999–2006) also monitored vitamin B12 status using the commercial BioRad Quantaphase II radioprotein-binding assay kit (Yetley 2011). No serum vitamin B12 measurements were carried out between 2006 and 2010. From 2011 to 2014, serum vitamin B12 was measured using a fully automated electrochemiluminescence immunoassay on the Roche cobas e immunoassay analyzer. Serum vitamin B12 concentrations did not change appreciably since the introduction of folic acid fortification (Pfeiffer 2007). Central 95% reference intervals (pmol/L) were wider for persons ages 60–69 years (152–1530) and ≥70 years (137–1720) than for younger age groups (ages 20–39 years: 167–840; 40–59 years: 160–1150) (Mineva 2019). The prevalence of vitamin B12 deficiency (serum B12 <148 pmol/L, serum/plasma MMA >376 nmol/L, and plasma tHcy >15 µmol/L) was stable at ~2–4% between NHANES 1999–2000 and 2013–2014, while insufficiency (serum B12 <221 pmol/L and serum/plasma MMA >271 nmol/L) showed a significant increasing linear trend from ~7% to ~10% (Pfeiffer 2026c).

tHCY and MMA. Plasma metabolite concentrations were also monitored during several years of the continuous survey (tHCY 1999–2006; MMA 1999–2004 and 2011–2014). tHCY data were generated using a commercial Abbott fluorescence polarization immunoassay kit. MMA data were generated first using a GC-MS and then an HPLC-MS/MS method; both methods generated comparable data (Mineva 2015). Circulating tHCY concentrations decreased by approximately 10% in a national sample of the U.S. population from pre-fortification to post-fortification (Pfeiffer 2008). The serum MMA central 95% reference intervals (nmol/L) were considerably narrower for a vitamin B12-replete subpopulation with normal renal function compared to the overall population. However, they were still age-dependent, with higher MMA concentrations in older persons (Mineva 2019).

3cB12. When data for all four biomarkers are available, this indicator is calculated using the equation 4cB12 = log10 [(holoTC × B12) / (MMA × tHCY)] - (age factor) (Fedosov 2013). Data for

serum B12 and plasma MMA and tHCY are available for three consecutive NHANES cycles (1999–2000, 2001–2002, and 2003–2004), while no data are available for holoTC. Therefore, a modified equation (Fedosov 2015) was used to calculate 3cB12 (B12, MMA, tHCY): the reference interval (2.5th and 97.5th percentile) for U.S. adults (≥20 y) was -0.538 to 1.60 (Mineva 2021). In covariate-adjusted models, 3cB12 was not associated with age. Adult females and B12 supplements users had higher 3cB12 levels, while adults with advanced chronic kidney disease had lower levels, regardless of race and Hispanic origin (Mineva 2021). Using the proposed cB12 cutoffs, 0.1% of U.S. adults had low (3cB12 <-2.5) and 2.7% had low or transitional (3cB12 ≤-0.5) vitamin B12 status (Mineva 2021). By comparison, the prevalence of low (or low-normal) status varied by the individual, conventional marker and traditional cutoff used: 2.2% (B12 <148 pmol/L), 13% (B12 148–222 pmol/L), 6.0% (MMA above age-specific cutoff: 250–320 nmol/L), and 8.4% (tHCY >13 µmol/L) (Mineva 2021). When comparing the diagnostic accuracy of conventional vitamin B12 status markers, singly or in combination, relative to 3cB12 as a reference, MMA showed a better performance than vitamin B12 or tHCY in U.S. adults with normal renal function (Mineva 2026). When using a single marker, MMA >376 nmol/L may be a useful cutoff for healthy populations with low (<5%) prevalence of inadequate vitamin B12 status to minimize false positives, whereas MMA >229 nmol/L may be preferred for screening higher-risk groups to detect more cases (Mineva 2026).

Multiple B-vitamin biomarkers. A multiple regression analysis of NHANES 2003–2006 examined the effects of sociodemographic (age, education, income, race-ethnicity, and sex) and lifestyle (alcohol consumption, body mass index, dietary supplement use, physical activity, and smoking) variables on the variability of B vitamin biomarkers. Together, these variables explained from 7% (vitamin B12) to 29% (tHCY) of the biomarker variability (Pfeiffer 2013). Age, sex, and race and Hispanic origin differences in B vitamin biomarker concentrations observed in crude univariate analysis remained significant after adjusting for sociodemographic and lifestyle variables. Use of dietary supplements and smoking were important correlates of these biomarkers (Pfeiffer 2013). A second multiple regression analysis of NHANES 2003–2006 showed that, after controlling for demographic variables, smoking, supplement use, fasting, inflammation, and renal function, fasting was associated with significantly higher serum folate (9.5%), RBC folate (4.9%), MMA (21%), PLP (9.3%), and 4PA (28.2%) and significantly lower tHCY (-2.5%) concentrations. Inflammation was associated with significantly higher RBC folate (5.5%) and significantly lower serum folate (-4.5%) and PLP (-28.5%) concentrations. Impaired renal function was associated with significantly higher serum folate (6.8%), RBC folate (8.5%), vitamin B12 (5.3%), tHCY (32.3%), MMA (42.3%), and

4PA (66.3%) concentrations (Haynes 2013). Pregnancy (in women ages 20–49 years) was associated with significantly higher serum folate (17.9%), RBC folate (26.2%), and 4PA (33.8%), and significantly lower vitamin B12 (-21.5%) and tHCY (-29.6%) concentrations (Haynes 2013). A third multiple regression analysis of NHANES 2003–2006 evaluated sociodemographic, lifestyle, and physiologic factors as potential confounders or effect modifiers of the relationship between biomarkers and intake. The investigation demonstrated that dietary supplement use explains more variance in serum folate, RBC folate, PLP, and B12 concentrations than 24-hour dietary intake from food only (Sternberg 2026).

For more information on B vitamins and related biochemical indicators, see the Institute of Medicine's Dietary Reference Intake reports (Institute of Medicine 1998) and fact sheets from the National Institutes of Health (NIH), Office of Dietary Supplements (https://ods.od.nih.gov/factsheets/list-VitaminsMinerals/). Narrative reviews for vitamins B6 and B12 as part of a broader panel of neglected micronutrients have been published (Brown 2025).

Data in the 2026 tables

Data presented are from univariate analysis that was not adjusted for demographic variables (e.g., age, sex, race and Hispanic origin) or other blood concentration determinants (e.g., dietary intake, supplement use, smoking, BMI). Data for the B-vitamin biomarkers were available from different NHANES cycles and have been generated using different methods. To allow for comparisons over time, we present method-adjusted data in tables or figures that cover a time period in which multiple methods were used. During time periods where only one method was used, we present the original method data. Table footnotes indicate whether original or adjusted method data are presented. Data from the 2017–2018 cycle are not included because selected subpopulations were surveyed (full sample for females ages 12–49 years and 1/2 subsample for males ages ≥1 years, females ages 1–11 years, and females ages ≥50 years). Data from the 2019–March 2020 cycle are also not included. Field operations were suspended in March 2020 due to the COVID-19 pandemic, and the resulting data were not considered nationally representative. NHANES resumed operations in August 2021, and the most recent cycle with publicly available data concluded in August 2023.

B vitamin biomarker	NHANES cycle	Method
RBC “total” folate	1999–2006 2007–2010, 2011–2018, Aug 2021-Aug 2023	BioRad radioprotein-binding assay Microbiologic assay
Serum “total” folate	1999–2006 2007–2010 2011–2018, Aug 2021-Aug 2023	BioRad radioprotein-binding assay Microbiologic assay HPLC-MS/MS
Serum 5-methyltetrahydrofolate	2007–2008, 2011–2018, Aug 2021-Aug 2023	HPLC-MS/MS
Serum folic acid	2007–2008, 2011–2018, Aug 2021-Aug 2023	HPLC-MS/MS
Serum tetrahydrofolate	2011–2018, Aug 2021-Aug 2023	HPLC-MS/MS
Serum 5-formyltetrahydrofolate	2011–2018, Aug 2021-Aug 2023	HPLC-MS/MS
Serum 5,10-methenyl-tetrahydrofolate	2011–2018, Aug 2021-Aug 2023	HPLC-MS/MS
Serum MeFox	2011–2018, Aug 2021-Aug 2023	HPLC-MS/MS
RBC “total” folate	Aug 2021-Aug 2023	HPLC-MS/MS
RBC 5-methyltetrahydrofolate	Aug 2021-Aug 2023	HPLC-MS/MS
RBC folic acid	Aug 2021-Aug 2023	HPLC-MS/MS
RBC tetrahydrofolate	Aug 2021-Aug 2023	HPLC-MS/MS
RBC 5-formyltetrahydrofolate	Aug 2021-Aug 2023	HPLC-MS/MS
RBC 5,10-methenyl-tetrahydrofolate	Aug 2021-Aug 2023	HPLC-MS/MS
RBC MeFox	Aug 2021-Aug 2023	HPLC-MS/MS
Serum pyridoxal-5’-phosphate	2005–2010	HPLC with fluorometric detection
Serum 4-pyridoxic acid	2005–2010	HPLC with fluorometric detection
Serum vitamin B12	1999–2006 2011–2014	BioRad radioprotein-binding assay Roche electrochemiluminescence immunoassay
Plasma total homocysteine	1999–2006	Abbott fluorescence polarization immunoassay
Plasma methylmalonic acid	1999–2004 2011–2014	GC-MS HPLC-MS/MS

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