Folic acid, riboflavin, thiamine, and vitamin B-6 status of a group of first-time blood donors

Christine K Booth, Therese Clark, and Anne Fenn

ABSTRACT  Reference intervals for long-term status measures of folate, riboflavin, thiamine, and vitamin B-6 were determined in a select group of adults. Reference subjects had no adverse medical history and did not use tobacco, alcohol, or nutritional supplements, and their diets met ≥70% of the Australian recommended dietary intake for nutrients. Red blood cell concentrations of thiamine and folate were measured by microbiological methods. Vitamin B-6 and riboflavin status were measured on the basis of the erythrocyte aspartate transaminase activity coefficient and erythrocyte glutathione reductase activity coefficient, respectively. A survey of first-time blood donors, which was conducted in Australia in 1995, revealed a significant prevalence of low red blood cell thiamine concentrations (13%) when compared with the calculated normal reference intervals. However, the most important finding in the survey was that the group of healthy, nonanemic adults (first-time blood donors) was found to have a median red blood cell folate concentration 24% below the median concentration of the carefully selected (non-supplemented) reference group. Plasma total homocysteine concentrations indicated folate deficiency in the reference group. Therefore, the 2.5th percentile cutoff for reference group red blood cell folate concentrations may have underestimated the prevalence of folate deficiency in the survey group. In each case, the method to determine long-term status rather than acute status was chosen, namely, measurement of red blood cell (RBC) total folate (4), and RBC cell total thiamine (5) concentrations, erythrocyte aspartate transaminase activation test (6), and the erythrocyte glutathione reductase activation test (7). These are measures that should not be influenced by the subject’s previous meal.

SUBJECTS AND METHODS

Subjects

First-time blood donors attending the Brisbane Queen Street Centre of the Red Cross Blood Transfusion Service in 1995 were invited to participate in a survey of B-group vitamin status. A smaller group was selected from staff and students of the Royal Brisbane Hospital and Queensland University of Technology for determination of normal reference intervals. The final data set included 1887 healthy adults, 111 in the reference group (53 men, 58 women) and 1776 in the survey group (851 men, 925 women). The mean age for the reference group was 39.2 y (range: 18–65 y) compared with 31.5 y for the survey group (range: 16–70 y). The age distribution of both groups was normal. There was no significant difference in the sex breakdown of

INTRODUCTION

In Australia, the fortification of food with vitamins and minerals is the subject of much debate. The Australia New Zealand Food Authority recognizes that there needs to be a sound rationale for the addition of vitamins and minerals to Australian foods and recommends that food fortification should occur only where there is an identified and proven public health and nutritional need. However, a recent inquiry by the Australia New Zealand Food Authority into vitamins and minerals highlighted the lack of Australian dietary and nutritional status data (1). Furthermore,
the 2 groups ($x^2 = 0.001, P = 0.972$). Although the difference in the mean ages of the 2 groups was significant ($P < 0.05$), the reference group was considered to be appropriate for the vitamins of interest in this study because no association between age and any of the vitamin measures was noted.

The experimental procedures were approved by the Human Bioethics Committee of the Queensland University of Technology. Written consent was obtained from each subject after the details of the study were explained to him or her.

Survey group

All potential blood donors were required to complete a questionnaire and undergo an interview to ascertain their suitability for blood donation. The information obtained is confidential and was not available for this survey. All subjects accepted for blood donation were considered healthy; subjects had good hemoglobin status and had not been using any medication long-term. Although social data were not collected in this survey, the experience of previous surveys conducted through the Blood Transfusion Service suggests that first-time donors include people from a wide range of educational backgrounds and occupations. Dietary intake data were not collected.

Reference group

Direct selection of reference subjects is the only method that agrees with the reference values concept recommended by the International Federation of Clinical Chemists (8). Subjects were asked to fast overnight for 9 h before donating a 20-mL blood sample. Subjects completed medical history and food-frequency questionnaires (150 foods), which were administered by an accredited, practicing dietitian. The food-frequency questionnaire had been validated against a 3-d dietary intake diary in a previous study (9). Nutrient intake was calculated by using the DIET/1 NUTRIENT calculation software (Xyris Software, Brisbane, Australia), which used the NUTTAB 90 database, a database of Australian foods. This database does not include values for folate or vitamin B-6. Those subjects accepted for inclusion in the reference group had no adverse medical history, had an acceptable weight-for-height (10), and were consuming diets providing ≥70% of the recommended dietary intake (RDI) for nutrients (11). Subjects were nonsmokers and took neither nutritional supplements nor medications within 2 wk of blood donation. Furthermore, subjects were social drinkers only, and did not drink alcohol within 48 h of blood donation. All reference subjects had normal biochemistry test results (iron studies, lipid profile, electrolytes, and liver-function tests). These biochemical measurements, which used standard methods, were performed by the Royal Brisbane Hospital Chemical Pathology Department. The vitamins (folate, vitamin B-6, thiamine, and riboflavin) were measured in blood collected from reference subjects, as was total homocysteine. Initially, 172 subjects were enrolled in the reference study and after processing the medical, biochemical, and dietary data, 111 subjects were accepted into the reference group.

Biochemical analysis

Vitamin B-6 and riboflavin status were assessed by use of a functional enzyme method (12). Vitamin B-6 (pyridoxal 5'-phosphate) acts as a coenzyme for the enzyme erythrocyte aspartate transaminase (EAST). The activity of this enzyme is increased by the addition in vitro of vitamin B-6. The EAST activity coefficient (EASTAC), expressed as a percentage, was calculated as the stimulated activity minus the unstimulated activity divided by the unstimulated activity times 100. In a similar fashion, the erythrocyte glutathione reductase activity coefficient (EGRAC), which was used as a measure of riboflavin status, was calculated by measuring the enzyme activity before and after in vitro addition of its coenzyme, flavin adenine dinucleotide. RBC folate and thiamine were measured by microbiological assay using chloramphenicol-resistant strains of bacteria: Lactobacillus casei (13) and L. fermentum (14), respectively. The assays are based on the nutritional need of L. casei for folate and of L. fermentum for thiamine. Total plasma homocysteine was defined as the sum of all homocysteine species in plasma, including homocysteine, homocystine, mixed disulfides, and protein-bound forms. All these forms were converted to homocysteine by reduction with sodium borohydride according to the method of Allena et al (15).

Quality-control material was prepared in batches by separating the RBCs from the blood of a single donor (normal control; supplied by the Red Cross Blood Transfusion Service, Queensland, Australia) and from pooled blood samples with low values (low control). Aliquots of the packed cells were stored at −70°C for 6 mo. An anemia control serum (Lyphochem Anemia Control; Bio-Rad, Anaheim, CA) was also used as quality-control material in the folate assay. Homocysteine reference material was supplied by the ERNDIM Foundation (Maastricht, Netherlands). Shewart mean and range plots were used for quality control (16). Control limits were calculated from data obtained after 5 batch analyses of the control material and were determined to provide an acceptable precision (probability of false rejection of 0.01).

A venous blood sample was drawn from each subject into a tube containing lithium heparin. After the plasma and buffy coat were removed, the RBCs were washed twice with isotonic saline solution. Aliquots of RBCs were diluted in sodium acetate buffer (0.2 mol/L), pH 5.0, for thiamine analysis and into 50-mmol/L buffer, pH 4.5, containing 1% (vol:vol) L-ascorbic acid and 15 mmol β-mercaptoethanol/L for folate analysis. The remaining cells were hemolyzed by 5-fold dilution into 0.2% (vol:vol) Triton X-100 and the cell debris was removed by centrifugation (1500 × g for 10 min at 4°C). Supernates, stored at −20°C, were later used for EASTAC and EGRAC assays.

Statistical analysis

Because many of the blood distributions were non-normal and could not be normalized by simple transformations, the data are presented as medians and percentiles. Wilcoxon’s rank-sum test for differences between medians was used. The Kolmogorov-Smirnov test was used for comparing all non-normal distributions. The cutoff points for the normal reference intervals were defined as being less than the 2.5th percentile or greater than the 97.5th percentile. Subjects were classed as either low, normal, or high, depending on whether they were below, within, or above the percentile cutoffs for the reference data, respectively. A chi-square goodness-of-fit test was performed to examine whether the survey data were outside the cutoffs in the same proportion as the reference data. Statistical analysis was performed by using SAS software (SAS Institute Inc, Cary, NC).

RESULTS

The normal reference intervals (2.5th percentile to 97.5th percentile) determined in the present study, reference intervals used
in 3 Australian hospitals, and clinically significant cutoffs from previously published reports are shown in Table 1. There were differences between the reference group and the survey group. The survey distributions for folate concentration and EASTAC differed from the reference data (Table 2 and Figure 1). In each case, the median values were significantly lower than those for the reference group (Wilcoxon test: folate, $P = 0.0001$; EASTAC, $P = 0.0002$). However, when the proportion of survey subjects outside the normal reference intervals was considered, only the prevalence of abnormal thiamine values was significantly different from that of the reference group (Table 3). The details of the distribution of measured homocysteine values for the reference group are shown in Figure 2. The central 95% of this distribution fell within 4.4–23.6 μmol/L. The median value was 11.0 μmol/L.

### DISCUSSION

The lack of reliable reference values and standardized analytic methods makes it difficult to assess vitamin status and to compare the status data reported by different surveys (22). Differences in analytic procedures and criteria for selection of the reference population can result in the calculation of differing reference intervals (Table 1). For example, supplementation with vitamin B-6 can lower the cutoff for EASTAC from <130% to <86% (20). The current study used strict selection criteria and reported normal reference intervals for the vitamin status of healthy subjects who consumed a diet (without nutritional supplements) that provided ≥70% of the Australian RDI for nutrients (11). This is consistent with the recommendations of the Scandinavian Committee on Reference Values (23).

In clinical practice, it is usual to compare an observed patient’s value with the corresponding normal reference interval (the central 95% of the reference population). Such a comparison conveys information about the similarity of the patient’s values to the given set of reference values. This contrasts with clinical decision cutoffs, which are based on the analysis of data from several population groups (healthy persons and patients with relevant diseases). Except for EASTAC, the normal reference intervals determined by the present study were consistent with clinical cutoffs described in the literature (Table 1).

The values of EASTAC that correspond with either optimal body stores of vitamin B-6 or to borderline deficiency are not well established. Gibson (24) referred to the >50% clinical cutoff proposed by Sauberlich et al (25), and Leklem (19) suggested a clinical cutoff of >80%. The different assay conditions used by laboratories may explain the lack of consensus regarding cutoffs for EASTAC. The cutoff proposed by Sauberlich et al (25) was revised to 130% by Bayouni and Rosalki (20) and confirmed by Mount et al (12). These authors optimized the substrate concentrations used in the automated assay. The assay conditions described by the latter authors were used in the present study. When the cutoff of >80% was used, >70% of both the reference and survey groups had abnormal EASTAC results. The literature suggests that such a high prevalence of vitamin B-6 deficiency in healthy subjects is unlikely. In fact, the reference group had a significantly higher median EASTAC value than the survey group, which indicates better vitamin B-6 status in the survey subjects (Table 2).

Possible folate, thiamine, vitamin B-6, and riboflavin deficiencies were identified in first-time blood donors (Table 3). For folate, vitamin B-6, and riboflavin, the prevalence of abnormal values was not significantly different from that of the reference group and the blood donors were therefore considered to have a low risk of these deficiencies.

First-time blood donors in the survey group could be at risk of thiamine deficiency. A significant proportion (13%) of the survey group conveyed low risk of these deficiencies.

### TABLE 1

Reference intervals for thiamine, folate, vitamin B-6, and riboflavin status measures

<table>
<thead>
<tr>
<th>Vitamin Measure</th>
<th>Group</th>
<th>Normal Reference Intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>This study</td>
<td>Other laboratories</td>
</tr>
<tr>
<td>Folate (nmol/L)</td>
<td>615</td>
<td>216</td>
</tr>
<tr>
<td>Thiamine (nmol/L)</td>
<td>315</td>
<td>200</td>
</tr>
<tr>
<td>EASTAC (%)</td>
<td>200–520</td>
<td>&lt;190</td>
</tr>
<tr>
<td>EGRAC (%)</td>
<td>&lt;190</td>
<td>&lt;120</td>
</tr>
</tbody>
</table>

1Except for folate, which was measured with an immunologic method at Royal Brisbane Hospital, assay methods were those used in the present study. Reference numbers in parentheses.

2Royal Brisbane Hospital, Pathology Department, Brisbane, Australia.

3Princess Alexandra Hospital, Brisbane, Australia.

4Royal Perth Hospital, Perth, Australia.

### TABLE 2

Distribution of each of the vitamin measurements for the reference and survey groups

<table>
<thead>
<tr>
<th>Vitamin Measure</th>
<th>Reference group</th>
<th>Survey group</th>
<th>Kolmogorov-Smirnov test (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate (nmol/L)</td>
<td>Median 615</td>
<td>216</td>
<td>1100</td>
</tr>
<tr>
<td>Thiamine (nmol/L)</td>
<td>315</td>
<td>200</td>
<td>520</td>
</tr>
<tr>
<td>EASTAC (%)</td>
<td>121</td>
<td>18.6</td>
<td>212</td>
</tr>
<tr>
<td>EGRAC (%)</td>
<td>14.7</td>
<td>0</td>
<td>47.6</td>
</tr>
</tbody>
</table>

1EASTAC, erythrocyte aspartate transaminase activity coefficient, which was calculated as the stimulated enzyme activity minus the unstimulated activity divided by the unstimulated activity multiplied by 100; EGRAC, erythrocyte glutathione reductase activity coefficient.
vey group had low RBC thiamine concentrations. Thiamine has been considered to be one of the marginally adequate nutrients in the Australian diet. Certainly, poor thiamine status has been identified in homeless people (26) and a high incidence of Wernicke-Korsakoff syndrome has been recorded (27). It was believed that thiamine enrichment of flour to the concentration of 6.4 mg/kg, which commenced in 1991, would alleviate the problem of thiamine status in the Australian population (28). The present finding suggests that further monitoring of the fortification program is needed.

Possibly the most important finding in the survey concerns folate status. Inadequate dietary intake of folate has been found to increase the risk of spina bifida and other neural tube defects (29) and the evidence is convincing that an increased intake of folate can prevent most neural tube defect cases. The official recommendation for Australian women at conception and for 4 wk thereafter is to consume 400 μg folate/d (30). Homocysteine, which has been identified as an independent risk factor for cardiovascular disease, is inversely related to folate status. Moderately elevated homocysteine concentrations may be the first biochemical marker of insufficient intracellular folate.

There is evidence that strongly suggests that the 2.5th percentile cutoff underestimates folate deficiency (31). A more appropriate cutoff for RBC folate in the population might be determined by using plasma total homocysteine as a functional measure of folate status and studies of the prevention of neural tube defects. Recent studies suggest that the cutoff for a healthy plasma homocysteine concentration (ie, minimal risk for coronary artery disease) may fall below 10–15 μmol/L (31–33). In the present study, 6.8% of first-time blood donors (n = 97) fell below the 2.5th percentile reference value for RBC folate of 220 nmol/L. This is clearly an underestimate of folate deficiency because it is likely that a large number of the reference subjects were folate deficient (15% of the reference group had homocysteine values >15 μmol/L and 55% of the reference group had homocysteine values >10 μmol/L; Figure 2). Furthermore, the median RBC folate concentration in first-time blood donors would need to be increased by 31% to have a median equal to that of the reference group (Table 2).

The Australian RDI for folate for men and women is 200 μg (11). The folate content of Australian foods, and consequently an estimation of the availability of folate in the Australian food supply, has not been described. Surveys, which have used several international food-composition tables, indicate that Australian

![FIGURE 1. Box and whisker plots of the erythrocyte aspartate transaminase coefficient (EASTAC) and folate data for the reference and survey groups. The box extends from the 25th percentile to the 75th percentile, with a horizontal line at the median (50th percentile). Whiskers extend down to the smallest value and up to the largest. Median values for EASTAC: reference, 121%; survey, 101%; and for folate: reference, 615 nmol/L; survey, 468 nmol/L.](image1)

![FIGURE 2. Frequency distribution of total plasma homocysteine concentrations for the reference group. The central 95% of the distribution fell within 4.4–23.6 μmol/L. The median value was 11.0 μmol/L.](image2)

### TABLE 3

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Low (%)</th>
<th>Normal (%)</th>
<th>High (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate</td>
<td>6.8</td>
<td>92.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Thiamine</td>
<td>13.0</td>
<td>80.3</td>
<td>6.7</td>
</tr>
<tr>
<td>EASTAC</td>
<td>—</td>
<td>94.6</td>
<td>5.4</td>
</tr>
<tr>
<td>EGRAC</td>
<td>—</td>
<td>90.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>(\chi^2)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate</td>
<td>5.12</td>
<td>0.077</td>
</tr>
<tr>
<td>Thiamine</td>
<td>14.11</td>
<td>0.001</td>
</tr>
<tr>
<td>EASTAC</td>
<td>22.55</td>
<td>0.117</td>
</tr>
<tr>
<td>EGRAC</td>
<td>0.00</td>
<td>0.953</td>
</tr>
</tbody>
</table>

Low, normal, and high were less than the 2.5th percentile of the reference group, between the 2.5th and 97.5th percentiles of the reference group, and greater than the 97.5th percentile, respectively. For the enzyme activity coefficients, EASTAC (35.5%) and EGRAC (193%), the 95th percentile was used for the high group because of the one-way test. EASTAC, erythrocyte aspartate transaminase activity coefficient, which was calculated as the stimulated enzyme activity minus the unstimulated activity divided by the unstimulated activity multiplied by 100; EGRAC, erythrocyte glutathione reductase activity coefficient.
women are at risk of poor folate intake, particularly during pregnancy (34, 35). The recent National Nutrition Survey (36) estimated the mean daily intake of folate by adult males to be 307 µg and by adult females to be 233 µg. This is considerably less than the daily folate intake of 350 µg estimated to be needed to maintain normal plasma homocysteine concentrations (37). The recent voluntary addition of folic acid to the Australian food supply (38), if widely adopted by the food industry, was expected to dramatically reduce the proportion of women with inadequate intake of folate (34). In light of the data presented here, this assumption appears optimistic. The 30% difference in median RBC folate concentration between a carefully selected reference group and healthy first-time blood donors could justify calling for mandatory folic acid fortification of food. The argument for mandatory folic acid fortification is boosted by the fact that, to date, it has been supplemental folic acid rather than dietary folate that has been proven to prevent neural tube defects. Presumably, this is because of the increased bioavailability of folic acid compared with the various forms of folate found in foods.

The finding of vitamin deficiency in apparently healthy Australian adults was unexpected because Australian dietary intake data suggest that this is a group at low risk for nutritional deficiency (39). However, previous work has shown that the use of food-composition data may lead to substantial over- or understimation of the intake of several micronutrients and that the calculated amount of a specific micronutrient consumed did not adequately predict status as measured by several biochemical indexes (40). Furthermore, nutritional status is dynamic and changes with dietary and lifestyle habits and physiologic state. For example, alcohol intake, cigarette smoking, use of oral contraceptives, and exercise training can increase the requirements for specific vitamins (41–43).

Biochemical evidence of thiamine, vitamin B-6, and folate deficiencies has been found in homeless people (26, 44) and in an Aboriginal community (45). This is the first survey to suggest that thiamine and folate deficiencies are prevalent in an apparently healthy population of Australian adults.

We conclude that the prevalence of specific vitamin deficiencies in apparently healthy Australian adults may be greater than suggested by national dietary intake data. We recommend ongoing monitoring of the thiamine and folic acid fortification programs. We recommend mandatory fortification of the Australian food supply with folic acid and that biochemical assessment of vitamin status, which can be performed reliably and more cheaply than food-consumption surveys, should be considered as a viable alternative or addition to future nutrition surveys in Australia.

We thank Ha Le for assisting in analysis of blood samples, the staff of the Red Cross Blood Transfusion Service for promoting the survey to donors, and the staff of the Royal Brisbane Hospital Chemical Pathology Department for performing the general biochemical analyses. The assistance of the Centre for Public Health Research, School of Public Health, Queensland University of Technology is acknowledged. Most importantly, our sincere gratitude is extended to the subjects who participated in the survey.

REFERENCES


