ABSTRACT

Background: Riboflavin status is commonly measured by the in vitro stimulation of erythrocyte glutathione reductase with flavin adenine dinucleotide and expressed as an erythrocyte glutathione reductase activation coefficient (EGRAC). However, this assay is insensitive to poor riboflavin status in subjects with glucose-6-phosphate dehydrogenase (G6PD) deficiency. Because G6PD deficiency is common in parts of the world where arboflavinosis is endemic, it is important to have a measure of riboflavin status that is unaffected by differences in G6PD status.

Objective: The objective was to further develop and validate a fluorometric assay for pyridoxamine phosphate oxidase (PPO) activity as a measure of riboflavin status.

Design: A fluorometric assay was optimized for the flavin-dependent enzyme PPO in erythrocytes. Hemolysates from a previous riboflavin intervention study (2- and 4-mg riboflavin supplements) were used to investigate the responsiveness of the method to changes in riboflavin intake.

Results: PPO activity and the PPO activation coefficient (PPOAC) were used to assess riboflavin status. Both PPO activity and PPOAC responded to riboflavin supplements (P < 0.01), but only PPO showed a dose response (P < 0.001). The change from baseline to after the intervention in PPOAC and PPO enzyme activity was significantly inversely correlated (P < 0.001). Both PPO activity and PPOAC were strongly correlated with EGRAC (P < 0.001). Additionally, both PPOAC and EGRAC showed a significant inverse correlation with dietary riboflavin intake (P < 0.01); PPO activity was positively correlated with riboflavin intake (P < 0.01).

Conclusion: PPO activity could be used as a biomarker for measuring riboflavin status, especially in populations with a high prevalence of G6PD deficiency. This trial is registered at www.isrctn.org as ISRCTN35811298.

INTRODUCTION

Riboflavin status is currently determined on the basis of 2 commonly used measurements: urinary riboflavin and erythrocyte glutathione reductase activation coefficient (EGRAC). Urinary riboflavin can be measured fluorometrically (1) and is a useful marker for optimum nutritional status either as total 24-h urinary riboflavin or when expressed as mmol/mol creatinine. However, this approach has limitations at lower intakes because of the sharp increase in excretion of riboflavin at intakes of ~1.0 mg/d (2).

Riboflavin status is more commonly measured by the in vitro stimulation of erythrocyte glutathione reductase (EC 1.6.4.2) with flavin adenine dinucleotide (FAD) and expressed as EGRAC. This method is used for the National Diet and Nutrition Surveys of the United Kingdom. However, this approach can also give anomalous results under certain circumstances. Thurnham (3) showed that this assay could not detect poor riboflavin status in subjects with glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) deficiency. It is known that this condition is associated with enhanced binding of FAD to erythrocyte glutathione reductase, which effectively masks riboflavin deficiency assessed as elevated EGRAC (4). Because G6PD deficiency is not uncommon in parts of the world where arboflavinosis is endemic, it is important to have a measure of riboflavin status that is unaffected by differences in G6PD status.

Pyridoxine (pyridoxamine) phosphate oxidase (PPO; EC 1.4.3.5) is an essential flavin mononucleotide (FMN)–dependent enzyme that plays a role in the conversion of the 5-phosphates of both pyridoxine and pyridoxamine to the predominant coenzyme pyridoxal phosphate (PLP), as was first shown with the purified enzyme from liver (5). Activity was subsequently shown in the soluble fraction of other tissues and in blood cells (6, 7). Studies in rats showed that the activity of this enzyme was sensitive to changes in riboflavin status and showed superior sensitivity to the conventional EGR assay (8, 9). Assays developed to measure PPO activity are based on fluorescence (10) and radioisotope (11) methods.

In a previous study (12), we measured PPO activity and EGRAC in the red blood cell lysates of 72 Gambian women. (Riboflavin deficiency is endemic in the rural population of the Gambia). The measurements were repeated after supplementation with either riboflavin (5 mg) or placebo for 6 wk. The riboflavin intervention group responded with an increase in PPO activity and a decrease in EGRAC. However, 3 women who were identified as being G6PD-deficient showed a marked increase in PPO activity after riboflavin supplementation, whereas their corresponding EGRAC values showed a much smaller increase.
response. The objectives of the present study were to further develop and optimize a fluorometric assay for PPO activity based on the method described in our earlier study (12), to examine the usefulness of an activation coefficient for this enzyme, and to investigate the responsiveness of these measurements to a riboflavin supplement of 2 or 4 mg/d for 8 wk in young women.

SUBJECTS AND METHODS

Materials

All chemicals were purchased from Fisher Scientific UK Ltd (Loughborough, United Kingdom) and Sigma Aldrich (Poole, United Kingdom) unless otherwise stated.

Subjects and blood sampling

Ethical approval was obtained from the University of Sheffield Ethics Committee (reference SM08ER15). The present study was conducted by using erythrocyte hemolysates obtained from a previous intervention study (FSA study NO5061) carried out over the period June 2006 to May 2008 in 145 young women (aged 19–25 y) recruited in Sheffield. The subjects were selected by screening for poor riboflavin status (EGRAC ≥ 1.40) and then randomly assigned to receive placebo, 2 mg riboflavin/d, or 4 mg riboflavin/d for 8 wk. Blood samples were collected before and after the period of supplementation. At the time of the study, the blood samples were collected in EDTA-containing tubes and centrifuged at 1000 × g at 4°C for 10 min. The plasma and the Buffy coat layer were removed, and the red blood cells were washed with phosphate-buffered saline (pH 7.4). The packed erythrocytes (hemolysates) were stored at −80°C and were not thawed before use in this PPO study. Twenty-three hemolysate samples were randomly selected from subjects in the placebo group, 23 samples from the 2-mg riboflavin/d group, and 22 samples from the 4-mg riboflavin/d group. Mean (±SD) baseline EGRAC values were 1.52 ± 0.15, 1.59 ± 0.18, and 1.59 ± 0.15, respectively.

EGRAC

EGRAC values for the collected samples had already been determined as part of the previous study. Erythrocyte glutathione reductase (EGR) is an FAD-dependent enzyme that catalyses the reduction of oxidized glutathione to its reduced form. The reaction also requires NADPH as an electron donor. FAD is a rate-limiting factor in this reaction. EGRAC is calculated from the ratio between the rate of disappearance of the NADPH in a sample to which an optimum amount of FAD has been added and to the same sample in which there has been no addition of FAD. The ratio is known as the activation coefficient.

\[
EGRAC = \frac{EGR \text{ activity with added FAD in vitro}}{EGR \text{ activity without added FAD in vitro}} \quad (1)
\]

The assay was carried out by using the Powers’ method (13, 14). Two 120-μL aliquots of hemolysates were pipetted into the sample cups of a comprehensive biochemical system (COBAS) rotor. To one aliquot, 20 μL phosphate buffer was added (unstimulated) and, to the other aliquot, 20 μL FAD (final concentration − 2.0 μmol/L) was added (stimulated). Sample cups were mixed and placed in an incubator at 37°C for 30 min. The rotor was then placed in a COBAS autoanalyzer (Roche, Rotkreuz, Switzerland), and the activity of glutathione reductase was measured. During the run, 90 μL oxidized glutathione (final concentration − 0.59 μmol/L) was added to 80-μL hemolysates, and the reaction was initiated by the addition of 70 μL NADPH (final concentration − 137 μmol/L). The oxidation of NADPH in the reaction system was monitored at 340 nm with automatic readings every 10 s for 5 min. EGRAC was then calculated by using Equation 1. The predetermined intrabatch CV for this method was <5%, and the interbatch CV was 1.7%.

Measurement of PPO activity

Principle

PPO, an FMN-dependent enzyme, catalyzes the oxidation of both pyridoxine phosphate and pyridoxamine phosphate (PMP), forming PLP. FMN acts as the immediate electron acceptor, and oxygen serves as an electron acceptor producing H2O2 in these reactions (5). See Equations 2 and 3.

\[
\begin{align*}
\text{Pyridoxine phosphate} & + O_2 \xrightarrow{\text{FMN}} \text{pyridoxal phosphate} + H_2O_2 \\
\text{Pyridoxine phosphate} & + O_2 \xrightarrow{\text{FMN}} \text{pyridoxal phosphate} \xrightarrow{\text{H}_2\text{O}_2 + \text{NH}_3}
\end{align*}
\]

PLP can subsequently be converted to pyridoxal, catalyzed by the enzyme pyridoxal phosphate phosphatase:

\[
\text{Pyridoxine phosphate} \xrightarrow{\text{phosphatase}} \text{pyridoxal}
\]

For the present study, a fluorometric method, based on the reaction of semicarbazide to produce a fluorescent semicarbazone of PLP that is excited at 380 nm and emits at 460 nm, was used (15). However, over this wavelength range, pyridoxal, which emits at 380 nm when excited at 320 nm, interferes with the measurement of PLP and, therefore, the assay system relies on the observation that both PLP and pyridoxal yield equal fluorescence intensities on a molar basis after 15 min of the reaction.

Method

The initial method used in this study was that described by Bates and Powers (12): 125 μL hemolysates was diluted with 0.133 mol potassium phosphate buffer/L (pH 8.0) to a final volume of 500 μL. 50 μL of this solution was withdrawn for the measurement of hemoglobin concentration, and 80 μmol pyridoxamine 5-phosphate/L was added to the remaining solution to give a final concentration of 8 μmol/L for exogenous substrate. The solution was then incubated for 2 h at 37°C, during which time endogenous PPO and pyridoxal phosphate phosphatase were active. The reaction was stopped by adding 500 μL of 10% (wt:vol) trichloroacetic acid (TCA). The precipitated protein was removed by centrifugation at 20,000 × g for 10 min at 4°C. The protein-free extract was transferred to a COBAS autoanalyzer sample cup, and 1 volume of sample was mixed with 3 volumes of buffer (pH 9.5; 32.5 g potassium hydrogen
carbonate/L and 24.2 g potassium carbonate/L) followed by 0.2 volumes 12% (wt:vol) semicarbazide hydrochloride. The development of the fluorescent adduct with pyridoxal and PLP was measured for 15 min at 37°C at the peak excitation wavelength of 360 nm and an emission peak at 450 nm. PLP at concentrations of 0.2, 0.4, and 0.8 μmol/L were used as a standard.

Stimulation of PPO by FMN

To determine whether PPO could be stimulated by the addition of the cofactor FMN in vitro, the following experiment was carried out. To 3 aliquots of 125 μL hemolysates, 25 μL FMN was added to make final concentrations of 0.08, 0.17, and 0.34 μmol/L, respectively. This was repeated with 0.133 mol potassium phosphate buffer/L (pH 8.0) replacing FMN. The hemolysates were incubated at 37°C for 30 min; 50 μL of 80 μmol pyridoxamine 5-phosphate/L was then added to each aliquot of hemolysate to give a final concentration of 8 μmol/L for exogenous substrate. After a 2-h incubation at 37°C, the reaction was stopped by adding 500 μL of 10% wt:vol TCA. To the hemolysate aliquots containing FMN, 25 μL potassium phosphate (pH 8.0) was added and to the FMN-free aliquots 25 μL FMN was added to ensure that the final concentration of FMN was identical in both groups because this might influence the fluorescence. The precipitated protein was removed by centrifugation at 20,000 × g for 10 min at 4°C. The protein-free extracts were transferred to a COBAS autoanalyzer, and the PLP and pyridoxal concentrations were measured as described previously. The PPO activation coefficient (PPOAC) was then calculated by using the following equation:

\[
PPOAC = \frac{\text{PPO activity with added FMN in vitro}}{\text{PPO activity without added FMN in vitro}}
\]

To determine whether there was any interference due to possible fluorescence produced by FMN, the process was repeated with 6 different hemolysate samples, with a final concentration of added FMN of 0.17 μmol/L. Three aliquots of each sample were split into 3 separate samples: to the first, FMN was added before the 30-min incubation; to the second, FMN was added after the reaction was stopped by TCA; and to the third, no FMN was added.

Hemoglobin concentration determination

Hemoglobin was measured with a cyanmethemoglobin technique by using a Randox Haemoglobin assay kit (Randox Laboratories Limited, Crumlin, United Kingdom) and a COBAS autoanalyzer.

Statistical analysis

Statistical analysis was carried out by using SPSS software (SPSS 13.0 for Windows; SPSS Inc, Chicago, IL). Data were confirmed as normally distributed by using the Kolmogorov-
Smirnov test. A paired $t$ test was used to compare baseline and postintervention variables. One-factor ANOVA was used to analyze the change in variables from baseline to after the intervention between the 3 intervention groups. A paired $t$ test was used to analyze PLP concentrations and PPOAC values for hemolysates incubated with and without FMN. Pearson’s correlation coefficient was used to analyze the correlation between variables. A $P$ value $<0.05$ was considered statistically significant.

RESULTS

Method development

First, to confirm that PPO activity could be detected in hemolysates and to investigate dose response, PPO activity was measured in 3 aliquots of the same hemolysates (75, 100, and 125 $\mu$L). The increase in fluorescence values over 15 min due to semicarbazone derivitization of PLP for the 3 hemolysate aliquots is shown in Figure 1. This finding confirms PPO activity, because fluorescence from the derivitized product of the enzyme reaction (PLP) is clearly visible. There was a dose-dependent relation between the final fluorescence and the volume of hemolysates.

Bates and Powers (12) reported that the fluorescence produced by pyridoxal increased without reaching a plateau, whereas the fluorescence due to PLP reached a plateau within 3 min; both products yielded equal fluorescence intensities on a molar basis after 15 min. Thus, the extent of oxidation (pyridoxal + PLP) could be estimated. To confirm this in the present study, 4 different concentrations of PLP and pyridoxal (0.1, 0.2, 0.4, and 0.8 $\mu$mol/L) were measured instead of a hemolysate, and the results are shown in Figure 2, A and B, respectively. As described by Bates and Powers (12), the fluorescence produced by pyridoxal increased progressively without reaching a plateau (Figure 2B); however, fluorescence due to PLP did reach a plateau (Figure 2A) between 5 and 10 min, depending on the concentration of PLP. As the concentration of either pyridoxal or PLP increased, the final fluorescence increased approximately linearly. PLP and pyridoxal exhibited equal fluorescence intensities on a molar basis ($8 \mu$mol/L) after 20 min rather than after 15 min, as observed in our earlier study, as shown in Figure 2C (12). The equal fluorescence intensity response was observed for each of the equimolar concentrations of PLP and pyridoxal used. In the present study, the formation of fluorescent semicarbazone derivatives was therefore monitored for 20 min.

Stimulation of PPO with FMN

To determine whether PPO could be stimulated by the addition of FMN in vitro, thereby offering potential for the development of a PPO activation coefficient (PPOAC), 125 $\mu$L of 6 different

### TABLE 1

Pyridoxal phosphate (PLP) concentrations and pyridoxamine phosphate oxidase activation coefficient (PPOAC) values for 6 different hemolysates incubated with and without flavin mononucleotide (FMN) $^a$

<table>
<thead>
<tr>
<th>Hemolysate</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLP concentration + FMN ($\mu$mol/L) $^2$</td>
<td>1.53</td>
<td>0.62</td>
<td>0.71</td>
<td>0.82</td>
<td>0.75</td>
<td>0.83</td>
</tr>
<tr>
<td>PLP concentration − FMN ($\mu$mol/L) $^4$</td>
<td>1.37</td>
<td>0.4</td>
<td>0.42</td>
<td>0.64</td>
<td>0.51</td>
<td>0.71</td>
</tr>
<tr>
<td>PLP concentration, FMN added after incubation ($\mu$mol/L) $^4$</td>
<td>1.33</td>
<td>0.47</td>
<td>0.42</td>
<td>0.58</td>
<td>0.48</td>
<td>0.67</td>
</tr>
<tr>
<td>PPOAC</td>
<td>1.12</td>
<td>1.55</td>
<td>1.69</td>
<td>1.28</td>
<td>1.47</td>
<td>1.17</td>
</tr>
</tbody>
</table>

$^a$ A paired $t$ test was used to analyze the data. There was no significant difference ($P = 0.42$) between the PLP concentrations of the samples with FMN added after the reaction had been stopped and the samples without FMN.

$^2$ To 125 $\mu$L hemolysates, 25 $\mu$L FMN was added to make a final concentration of 0.17 $\mu$mol/L before incubation at 37°C for 30 min.

$^4$ To 125 $\mu$L hemolysates, 25 $\mu$L 0.133 mol potassium phosphate buffer/L (pH 8.0) was added instead of FMN before incubation at 37°C for 30 min.

$^6$ To 125 $\mu$L hemolysates, 25 $\mu$L 0.133 mol potassium phosphate buffer/L (pH 8.0) was added instead of FMN before incubation at 37°C for 30 min. After the subsequent derivitization reaction was stopped by trichloroacetic acid, FMN was added so that FMN was present in the final reaction mixture.

### TABLE 2

Baseline and postintervention erythrocyte glutathione reductase activation coefficient (EGRAC) values for the 0-, 2-, and 4-mg/d riboflavin groups $^a$

<table>
<thead>
<tr>
<th>Riboflavin intake</th>
<th>Baseline</th>
<th>After intervention</th>
<th>After intervention − baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg/d ($n = 23$)</td>
<td>1.52 ± 0.15</td>
<td>1.50 ± 0.19</td>
<td>−0.03 ± 0.14</td>
</tr>
<tr>
<td>2 mg/d ($n = 21$)</td>
<td>1.59 ± 0.18</td>
<td>1.34 ± 0.12 $^2$</td>
<td>−0.25 ± 0.17 $^4$</td>
</tr>
<tr>
<td>4 mg/d ($n = 22$)</td>
<td>1.59 ± 0.15</td>
<td>1.25 ± 0.13 $^2$</td>
<td>−0.33 ± 0.18 $^4$</td>
</tr>
</tbody>
</table>

$^a$ All values are means ± SDs. One-factor ANOVA was used to analyze the change in variables from baseline to after the intervention between the 3 intervention groups.

$^2$ Significantly different from baseline, $P < 0.001$.

$^4$ Significantly different from the 0-mg/d group, $P < 0.001$. 

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TABLE 3
Baseline and postintervention pyridoxamine phosphate oxidase (PPO) enzyme activity for the 0-, 2-, and 4-mg/d riboflavin groups

<table>
<thead>
<tr>
<th>Riboflavin intake</th>
<th>PPO activity at baseline</th>
<th>PPO activity after intervention</th>
<th>PPO activity (after intervention – baseline)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol · h⁻¹ · g Hb⁻¹</td>
<td>nmol · h⁻¹ · g Hb⁻¹</td>
<td>nmol · h⁻¹ · g Hb⁻¹</td>
</tr>
<tr>
<td>0 mg/d (n = 23)</td>
<td>5.54 ± 3.46</td>
<td>6.22 ± 4.38</td>
<td>0.68 ± 2.25</td>
</tr>
<tr>
<td>2 mg/d (n = 23)</td>
<td>5.96 ± 2.92</td>
<td>8.76 ± 3.78</td>
<td>2.80 ± 2.89</td>
</tr>
<tr>
<td>4 mg/d (n = 22)</td>
<td>5.84 ± 3.00</td>
<td>12.37 ± 4.06</td>
<td>6.52 ± 2.83</td>
</tr>
</tbody>
</table>

1 All values are means ± SDs. Hb, hemoglobin. One-factor ANOVA was used to analyze the change in variables from baseline to after the intervention between the 3 intervention groups.
2 Significantly different from baseline, P < 0.001.
3 Significantly different from the 0-mg/d group. 4Significantly different from the 2-mg/d group, P < 0.001.

TABLE 4
Baseline and postintervention pyridoxamine phosphate oxidase activation coefficient (PPOAC) values for the 0-, 2-, and 4-mg/d riboflavin groups

<table>
<thead>
<tr>
<th>Riboflavin intake</th>
<th>PPOAC</th>
<th>PPOAC</th>
<th>PPOAC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>After intervention</td>
<td>After intervention – baseline</td>
</tr>
<tr>
<td>0 mg/d (n = 22)</td>
<td>1.36 ± 0.20</td>
<td>1.34 ± 0.23</td>
<td>−0.02 ± 0.23</td>
</tr>
<tr>
<td>2 mg/d (n = 22)</td>
<td>1.40 ± 0.15</td>
<td>1.25 ± 0.15²</td>
<td>−0.15 ± 0.19</td>
</tr>
<tr>
<td>4 mg/d (n = 22)</td>
<td>1.46 ± 0.29</td>
<td>1.19 ± 0.20²</td>
<td>−0.27 ± 0.26²</td>
</tr>
</tbody>
</table>

1 All values are means ± SDs. One-factor ANOVA was used to analyze the change in variables from baseline to after the intervention between the 3 intervention groups.
2 Significantly different from baseline, P < 0.001.
3 Significantly different from the 0-mg/d group, P = 0.005.

hemolysates was incubated with and without 0.17 µmol FMN/L (final concentration), and PLP and pyridoxal were measured. This concentration was selected because it was found to be the optimal concentration for stimulation of PPO (data not shown). PLP concentrations with and without FMN are shown for the 6 samples in Table 1. The PLP concentrations were clearly higher for the samples with added FMN, which indicated that the PPO enzyme could be stimulated by the addition of FMN. To determine whether there was any interference due to the fluorescence of FMN, the 6 hemolysates were incubated without FMN, but after the derivitization reaction had been stopped by TCA, FMN was added so that FMN would be present in the final reaction mixture. As shown in Table 1, there was no significant difference (P = 0.42) between the PLP concentrations of the samples with FMN added after the reaction had been stopped and the samples without FMN.

PPO activity and PPOAC—final protocol

After it was shown that PPO could be stimulated by FMN, a final method was adopted for the determination of PPO activity relative to hemoglobin. Two aliquots of packed cell hemolysates (125 µL) were incubated at 37°C for 30 min: to one aliquot, 25 µL FMN were added to make the final concentration of 0.17 µmol/L, and to the second, 25 µL potassium phosphate buffer were added instead of FMN. Potassium phosphate buffer (300 µL of 0.133 mol/L; pH 8.0) and pyridoxamine 5-phosphate (50 µL of 80 µmol/L) were added to give a final concentration of 8 µmol/L. After a 2-h incubation at 37°C, the reaction was stopped by adding 500 µL of 10% (wt:vol) TCA. The precipitated protein was removed by centrifugation at 20,000 × g for 10 min at 4°C. The protein-free extract was transferred to the sample cup of a COBAS autoanalyzer, which was programmed to mix 1 volume of sample with 3 volumes of buffer (pH 9.5; 32.5 g potassium hydrogen carbonate/L and 24.2 g potassium carbonate/L) followed by 0.2 volumes of 12% (wt:vol) semicarbazide hydrochloride. The development of the fluorescent adduct with pyridoxal and PLP was measured for 20 min at 37°C at the peak excitation wavelength of 360 nm and emission peak at 450 nm. PLP (0.2, 0.4, and 0.8 µmol/L) was used as a standard.

The samples were run in duplicate, and the PLP concentrations of FMN-stimulated and -unstimulated samples were used to calculate PPOAC. The unstimulated hemolysates were used to determine PPO activity relative to hemoglobin. The hemoglobin concentration was measured in the hemolysates, and PPO activity was expressed as nmol PLP · h⁻¹ · g hemoglobin⁻¹.

To monitor precision, 125 µL of an in-house quality control (QC) hemolysate was run at the beginning and end of each sample run (6 samples in duplicate per run). The intrabatch CV was calculated by running 10 QC aliquots before any samples were run. The interbatch CV was calculated from the mean of the 2 QC values from each of 12 runs over the duration of the study. The intrabatch CVs for PPO activity before and after the addition of FMN were 1.24% and 8.7%, respectively; the equivalent interbatch CVs were 10.9% and 10.2%. The intrabatch and interbatch CVs for PPOAC were 9.9% and 4.6%, respectively.

Performance of the PPOAC method in test samples and comparison with EGRAC

Subject characteristics

In total, 68 subjects’ hemolysates were selected for the study (23 from the placebo group, 23 from the 2-mg/d group, and 22
from the 4-mg/d group), and the mean (± SD) age of the subjects was 21.4 ± 2.0 y.

Comparison of baseline and postintervention EGRAC values in hemolysate samples

The mean (± SD) baseline EGRAC of the entire sample was 1.56 ± 0.16, which reflected the poor riboflavin status of the recruited subjects (Table 2). There was a significant decrease in EGRAC from baseline to after the intervention for the 2-mg/d and the 4-mg/d groups (P = 0.002 and P < 0.001, respectively). There was no significant difference between the change in response to 2 and 4 mg/d, but both groups were significantly different from the placebo group (P < 0.001).

Comparison of baseline and postintervention PPO activity

Baseline and postintervention PPO activity are shown in Table 3. In both the 2- and 4-mg/d groups there was a significant increase in PPO activity after the intervention (P < 0.001), but
there was no significant change in the placebo group. The increase in enzyme activity in the 4-mg/d group was significantly greater than in the 2-mg/d group ($P < 0.001$), and the changes in response to both supplements were significantly greater than in the placebo group.

Comparison of baseline and postintervention PPOAC

Baseline and postintervention PPOAC values are shown in Table 4. Both the 2- and 4-mg/d supplements elicited a significant reduction in PPOAC ($P = 0.002$ and $P < 0.001$, respectively). The magnitude of the change in PPOAC between baseline and after the intervention was not significantly different between the 2 doses; the PPOAC change in response to the 4-mg dose was significantly different from that to the placebo ($P = 0.005$).

Relation between PPO activity and PPOAC

A significant and strong inverse correlation between PPOAC and PPO enzyme activity (all the hemolysate samples) is shown in Figure 3A ($r = -0.65$, $P < 0.001$). There was also a significant and strong inverse correlation between the change from baseline to after the intervention in PPOAC and PPO enzyme activity (all the hemolysates) as shown in Figure 3B ($r = -0.59$, $P < 0.001$).

Relation between PPO activity and EGRAC

A significant positive correlation between baseline EGRAC and baseline PPO activity ($r = 0.41$, $P = 0.001$), postintervention EGRAC and PPO activity ($r = 0.57$, $P < 0.001$), and combined baseline and postintervention EGRAC and PPO activity ($r = 0.56$, $P < 0.001$) is shown in Figure 4, A–C, respectively.

Relation between biomarkers of riboflavin status and dietary riboflavin intake

To examine how well each of the biomarkers of riboflavin status reflected riboflavin intakes, baseline dietary intake data from the original riboflavin supplementation study were used. Subjects had been asked to complete a 4-d food diary (including 1 weekend day) before the supplementation period. The diaries included detailed instructions, and food portion booklets based on the UK Ministry of Agriculture, Food and Fisheries food atlas were provided to assist with estimating portion sizes. The data

FIGURE 5. Correlation between baseline erythrocyte glutathione reductase activation coefficient (EGRAC) and pyridoxamine phosphate oxidase (PPO) activity (A; $n = 66$), postintervention EGRAC and PPO activity (B; $n = 68$), and combined baseline and postintervention EGRAC and PPO activity (C; $n = 134$). Hb, hemoglobin. Pearson’s correlation coefficients were used to analyze the correlation between variables.
from the food diaries were analyzed for micronutrient and macronutrient contents by using Windiets Research software (Robert Gordon University, Aberdeen, United Kingdom).

The relation between dietary riboflavin intake and biomarkers of riboflavin status was investigated. A significant inverse correlation between baseline EGRAC and dietary riboflavin \((r = 0.38, P = 0.003)\) is shown in Figure 6A. A significant inverse correlation between baseline PPOAC and dietary riboflavin intake \((r = 0.42, P = 0.002)\) is shown in Figure 6B. A significant positive linear correlation between baseline PPO activity and dietary riboflavin intake \((r = 0.35, P = 0.003)\) is shown in Figure 6C.

DISCUSSION

The present study has reported on the development and validation of a sensitive fluorometric method for the measurement of PPO activity, which can be used to assess riboflavin status. Two indexes, PPO enzyme activity (expressed as nmol PLP formed per hour, per gram of hemoglobin) and PPOAC, were used to assess riboflavin status. Of the 2 indexes, PPO enzyme activity was highly responsive to 2 levels of riboflavin supplements and showed a dose-response effect, whereas PPOAC decreased significantly in response to both riboflavin supplements but did not show a dose-response effect.

As anticipated, PPOAC showed a strong correlation with PPO activity, but the latter showed a much greater proportional change than did PPOAC in response to riboflavin supplementation. A change in PPOAC from \(\approx 1.0\) to 1.5 was equivalent to a change in PPO enzyme activity of from \(\approx 2.5\) to 17.5 nmol \(\cdot\) h\(^{-1}\) \cdot g hemoglobin\(^{-1}\). Additionally, PPOAC showed a strong positive correlation and PPO a strong negative correlation with the conventional measure of riboflavin status, EGRAC. Both activation coefficients as well as PPO activity were significantly correlated with dietary riboflavin intake.

Currently, the most frequently used method of measuring riboflavin status in humans is EGRAC, but, being an activation coefficient, it has a lower limit (of 1.0) and is a poor measure of changes in dietary riboflavin in riboflavin-replete individuals. This limits its potential as a biomarker for optimum nutritional status (16). It is however, favored over the measurement of EGR activity per se because the activation coefficient is unaffected by differences in the absolute amount of the apoenzyme. The same can be said of PPOAC and PPO. EGRAC and PPOAC behaved similarly in that, whereas they both decreased in response to 2 levels of riboflavin supplement, neither showed a dose response. EGRAC has been shown in many studies to be very sensitive to changes in riboflavin intake (17, 18), and in the present study it was more responsive than PPOAC to a 2-mg/d supplement of riboflavin. The fall from baseline to postintervention in EGRAC

![Figure 6](https://academic.oup.com/ajcn/article-abstract/90/5/1151/4598076)
was ≈16%, compared with a fall of 11% in PPOAC. This suggests that FMN-dependent PPOAC normalizes less readily than FAD-dependent EGRAC in response to an increase in riboflavin intake, which might make it more useful for evaluating marginal riboflavin status.

Studies in animals have shown that enzymes of flavin metabolism show a differential sensitivity to changing dietary riboflavin. Fass and Rivlin (19) reported that riboflavin deficiency has selective effects on enzymes involved in riboflavin metabolism, including flavokinase (catalyzes conversion of riboflavin to FMN), FAD pyrophosphorylase (converts FMN to FAD), and FMN phosphatase (degrades FMM to riboflavin). In riboflavin-deficient rats, flavokinase activity was depressed to 60% of normal, FAD pyrophosphorylase was elevated to 150% of normal, and FMN phosphatase was unaffected. These data are compatible with an adaptive mechanism that conserves FAD by sacrificing FMN when dietary riboflavin is low. Indeed, Prentice et al (20) showed that tissue FAD was conserved at the expense of tissue FMN during riboflavin depletion in rats. PPO activity might therefore be expected to fall before any effect on EGR, when riboflavin intakes fall, with an associated increase in the activation coefficient—PPOAC. This might explain the smaller improvement in PPOAC than in EGRAC in response to riboflavin supplements.

Thus, PPO or PPOAC offer alternatives to EGR or EGRAC as biomarkers of riboflavin status. The strongest case for using EGR/PPOAC in preference to EGR/EGRAC is that EGRAC is an unreliable indicator of riboflavin status in populations with a high prevalence of G6PD (12). G6PD deficiency appears to lead to an enhanced binding of FAD to the apoenzyme, such that even when riboflavin intakes are low, EGRAC values are within the normal range (3–5). The effect of G6PD deficiency on EGRAC is observed even in heterozygotes (21). G6PD deficiency is the most common human enzyme defect (17), and it is estimated that ≥400 million people worldwide could carry a mutation in the G6PD gene, causing deficiency (22). The highest prevalences of G6PD deficiency are reported in Africa, the Middle East, Southern and Eastern Asia, and Southern Europe; however, because of increased migration there is an increasing prevalence in North and South America and in parts of northern Europe (23). G6PD deficiency occurs in ≈10% of African Americans (24). Additionally, in our earlier study (12), we reported that EGRAC was relatively unresponsive to riboflavin supplements in subjects with G6PD, whereas PPO activities increased, although the data were limited to only a few subjects and PPOAC was not measured.

In conclusion, the present study describes the further development and validation of a fluorometric assay to measure PPO activity as an index of riboflavin status. PPO and PPOAC are both responsive to riboflavin supplements, and there is some evidence to suggest that PPOAC might be more useful than EGRAC over a wider range of riboflavin intakes. PPO/PPOAC might be preferable to EGR/EGRAC in populations in which there is a high prevalence of G6PD deficiency.

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