GeneScan profiles (Fig. 1) showed that DNA extracted from both these phenotypically benign lesions had a detectable monoclonal peak, indicating the presence of a clonal T-cell population. Moreover, these monoclonal peaks were identical in base pair size to the diagnostic monoclonal peak detected in the sample that established T-cell malignancy 8 years later.

This study validates the usefulness of TCRγ gene rearrangements in the differentiation of benign vs malignant cutaneous T-cell lymphoproliferative disorders. Furthermore, it illustrates the potential of the method to detect a clonal genetic fingerprint in cutaneous lesions that, at the time of presentation, do not show unequivocal clinical or pathologic signs of future malignant transformation. The diagnosis and monitoring of T-cell cutaneous lymphoproliferative disorders with a molecular approach similar to the one validated by us may have important diagnostic, prognostic, and therapeutic implications.

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References

Pyridoxal Phosphate Decreases in Plasma but not Erythrocytes during Systemic Inflammatory Response, Dinesh Talwar,1 Tara Quasim,2 Donald C. McMillan,2 John Kinsella,3 Cathy Williamson,1 and Denis St. J. O’Reilly1 (1 Department of Biochemistry, 2 University Department of Surgery, and 3 University Department of Anaesthesia, Royal Infirmary, Glasgow G31 2ER, United Kingdom; *author for correspondence: fax 44-0141-553-1703, e-mail dtalwar@gri.biochem.org.uk)

Vitamin B6 (active 3 hydroxy-2-methylpyridine derivatives) is an essential precursor of pyridoxal (PL) and pyridoxamine phosphate coenzymes of a wide variety of enzymes of intermediary metabolism (1). In plasma, pyridoxal 5'-phosphate (PLP) is the major form, whereas PLP and pyridoxamine 5'-phosphate (PMP) predominate in the cell. The most widely used method to detect vitamin B6 deficiency is the erythrocyte aspartate aminotransferase activation assay (2–5). As this test is a functional rather than a direct measurement of PLP status, it may be affected by factors other than PLP deficiency (2, 3). The plasma PLP concentration is considered one of the better indicators of vitamin B6 status (1, 6–9) and is reported to be well correlated with tissue PLP concentrations (8). However, vitamin concentrations in blood cells tend to be a better marker of cellular stores (10). We describe a simple, robust, reversed-phase HPLC method with precolumn derivatization using semicarbazide (11–13) that is suitable for the simultaneous measurement of PLP, 4-pyridoxic acid (PA), and PL in plasma and red cells and application of the assay in healthy individuals, patients with chronic disease, and critically ill patients requiring intensive care.

Venous blood samples (EDTA) and packed red cells for population reference values were obtained from apparently healthy individuals (laboratory staff and those attending a cardiovascular risk clinic). Samples were obtained from a group of chronically ill medical and surgical patients with the potential for vitamin deficiencies (60% with short bowel syndrome and 35% with either chronic liver or renal disease) and from a critically ill group of medical and surgical patients admitted to the intensive care unit (30% with pancreatitis, 30% with trauma, 30% with organ failure). The study was approved by the local ethics committee of Glasgow Royal Infirmary, and all patients gave informed consent.

PLP and PL in blood were measured as their semicarbazone derivatives, pyridoxal 5'-phosphate semicarbazone (PLPSC) and pyridoxal semicarbazone (PLSC), respectively (12, 13). The assay was optimized with respect to derivatization, separation, and detection. Derivatization was carried out as follows: 500 μL of plasma, calibra-
tor, quality controls, or diluted hemolysate (300 μL of red cells + 700 μL of water) and 40 μL of derivatizing agent (containing 250 g/L semicarbazide and glycine) were combined, vortex-mixed, and left in the dark at room temperature for 30 min. The mixtures were then deproteinated with 40 μL of 700 g/L perchloric acid, vortex-mixed for 1 min, and centrifuged for 10 min (1000g). Each supernatant (300 μL) was stabilized by the addition of 30 μL of 250 g/L NaOH (final pH between 3.0 and 5.0), and 50 μL was injected on the HPLC column via an autosampler (Waters).

HPLC separation was carried out using a Luna C 18 reversed-phase column [250 × 4.6 mm (i.d.); 5-μm bead size; protected with a 3 × 4 mm guard column] and an isocratic filtered mobile phase consisting of 60 mmol/L disodium hydrogen phosphate containing 95 mL/L methanol and 400 mg/L EDTA (disodium salt), adjusted to pH 6.8 with concentrated phosphoric acid. The flow rate was 1.5 mL/min. PLPSC and PLSC were detected using a programmable fluorescence detector (Waters). Quantification was by external standardization with a single-concentration plasma calibrator (Chromsystems), using peak heights. Red cell PLP, PA, and PL concentrations were related to hemoglobin (pmol/g Hb). There was no significant loss of PLP, PA, or PL at room temperature from derivatized plasma or red cells over 48 h. The performance of the method was monitored by taking part in the External Quality Assurance scheme for B vitamin (Dusseldorf, Germany).

Albumin and C-reactive protein concentrations in plasma were measured on an automated analyzer (ADVIA 1650; Bayer).

Data from the reference and chronically and critically ill groups are presented as median and range, and ANOVA (Kruskal-Wallis) was carried out. Correlations were carried out using the Spearman rank correlation (SPSS Inc.).

The chromatographic profiles corresponding to a derivatized plasma and a red cell hemolysate extract are shown in Fig. 1. The PLPSC, PA, and PLSC peaks were well resolved with K’ values of 4.7, 11.6, and 19.8, respectively. The peaks were identified by comparing peak retention times with those of PLP, PA, and PL aqueous calibrators after derivatization. For recovery studies, aliquots of a pooled plasma and red cell sample were analyzed both before and after the addition of 40 and 80 nmol/L of PLP, PA, and PL. The recoveries were >90% for all analytes. The within-batch imprecision (CV; n = 10) for PLP, PA, and PL in plasma and red cells was <5%, 12%, and 5%, respectively, from same-day analyses of quality-control material. The between-batch imprecision (n = 17) for PLP, PA, and PL in plasma and red cells was <7%, 14%, and 8%, respectively, from analysis of an aliquoted pooled red cell sample (stored at −70°C) analyzed over 3 months. The method was linear up to at least 1000 nmol/L for PLP, PA, and PL. The limits of detection, defined as three times the baseline noise, were 2.1 nmol/L for PLP, 1.0 nmol/L for PA, and 2.8 nmol/L for PL. The limits of quantification, defined as 10 times the signal-to-noise ratio, were 5.8 nmol/L for PLP, 2.5 nmol/L for PA, and 6.5 nmol/L for PL.

Previous methods have used pre- or postcolumn derivatization of PLP to its fluorescent derivative with use of semicarbazide, cyanide, or bisulfite as derivatization agents (2, 14–20). The major disadvantage of postcolumn derivatization procedures is that because of the photosensitivity of PLP, significant losses of PLP may occur during sample processing and analysis unless these steps are carried out away from ultraviolet light (14, 20). In contrast, we found the precolumn semicarbazide derivatization simple to perform, reliable, and with adequate sensitivity and precision for the routine measurement of PLP in plasma and red cells; it compared favorably with the methods reported by Vuilleumier et al. (2) and Millart et al. (15).

We measured PLP, PA, and PL concentrations in plasma and red cells in healthy individuals and patients with chronic and critical illness (Table 1). In healthy individuals, the geometric mean (95% reference interval) for PLP was 56 (21–138) nmol/L in plasma and 410 (250–680) pmol/g Hb in red cells, and plasma PLP
concentrations correlated with PA ($r^2 = 0.31; P < 0.0001$) and PL ($r^2 = 0.49; P < 0.0001$). However, in red cells PLP concentrations correlated with PL ($r^2 = 0.55; P < 0.0001$) but not with PA and was strongly positively correlated with PLP concentrations in the plasma of healthy individuals ($r^2 = 0.810; P < 0.0001$).

PLP concentrations in the critically ill group (Table 1) tended to be lower in plasma ($P = 0.03$) and red cells ($P = 0.05$) than in the reference population, but they remained significantly correlated ($r^2 = 0.412; P < 0.0001$), and assessment of vitamin B$_6$ status (plasma and red cell PLP) agreed in 28 of the 29 patients. In contrast, in the critically ill group with evidence of systemic inflammatory response and hypoalbuminemia (Table 1), plasma PLP was significantly lower ($P < 0.001$) and erythrocyte PLP was higher ($P = 0.023$) than in the reference population. Moreover, plasma and erythrocyte PLP concentrations were only weakly correlated ($r^2 = 0.358; P = 0.009$), and plasma PLP concentrations were below the reference interval in 12 of the 18 patients. However, the majority of the critically ill patients had erythrocyte PLP concentrations within the reference interval.

The reference intervals for PLP, PA, and PL in plasma were in good agreement with data reported by others (15–18). PLP concentrations in red cells were adjusted to Hb rather than to volume of packed red cells because accurate pipetting of packed red cells is difficult and affects precision. The strong correlation between plasma and red cell PLP concentrations in the reference population suggests that the latter are valid indicators of vitamin B$_6$ status and that either plasma or red cell PLP measurements can be used as markers of vitamin B$_6$ status. This is consistent with the concordance between plasma and red cell PLP concentrations in the critically ill group. In contrast, in the critically ill group there was little concordance between plasma and red cell PLP concentrations. A possible explanation for these results is that the abnormally low plasma PLP concentrations are related to recent poor nutritional intake, whereas the red cell PLP concentrations reflect vitamin B$_6$ status at the time of red blood cell synthesis. An alternative explanation is redistribution of plasma PLP (the majority of which is bound to albumin) during a pronounced systemic inflammatory response (21). Indeed, the plasma PLP results obtained in the present study are consistent with those reported previously after elective orthopedic surgery (21). Moreover, given that blood samples in this group were taken within 5 days of admission to the intensive care unit and that it is routine practice to give vitamin B supplementation as soon as practicable, the lack of concordance between plasma and red cell PLP concentrations in these patients is likely to be attributable to the low plasma concentrations. However, the present study does not provide a definitive explanation of the results obtained in the critically ill group.

From the results of the present study we conclude that measurement of plasma PLP for assessing vitamin B$_6$ status in patients with a systemic inflammatory response (as evidenced by an increased C-reactive protein concentration) may be misleading. In contrast, red cell PLP concentrations do not decrease in the presence of a systemic inflammatory response and therefore may be useful in differentiating true from apparent vitamin B$_6$ deficiency in patients with a systemic inflammatory response.

### Table 1. Vitamin B$_6$ status in a reference population and chronically and critically ill patients.

<table>
<thead>
<tr>
<th></th>
<th>Reference population (n = 126)</th>
<th>Chronically ill (n = 29)</th>
<th>Critically ill (n = 18)</th>
<th>P, ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, <em>a</em> years</td>
<td>53 (31–73)</td>
<td>51 (17–73)</td>
<td>60 (23–82)</td>
<td>0.770</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>67/59</td>
<td>13/16</td>
<td>10/8</td>
<td>0.684</td>
</tr>
<tr>
<td>C-reactive protein, <em>a</em> mg/L</td>
<td>&lt;6</td>
<td>8 (&lt;6 to 9)</td>
<td>130 (38–272)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Albumin, <em>a</em> g/L</td>
<td>43 (38–49)</td>
<td>39 (36–47)</td>
<td>22 (9–30)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma PLP, <em>a</em> nmol/L</td>
<td>52 (19–194)</td>
<td>43 (11–84)</td>
<td>15 (&lt;3 to 54)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Red cell PLP, <em>a</em> pmol/g Hb</td>
<td>391 (234–815)</td>
<td>360 (227–677)</td>
<td>579 (161–1539)</td>
<td>0.011</td>
</tr>
<tr>
<td>Plasma PLP/Red cell PLP*</td>
<td>0.13 (0.08–0.25)</td>
<td>0.12 (0.05–0.95)</td>
<td>0.03 (0.01–0.11)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Median (range).

### References


Stabilization of Homocysteine in Unseparated Blood over Several Days: A Solution for Epidemiological Studies, Sarah Clark,* Linda D. Youngman, Joan Sullivan, Richard Peto, and Rory Collins (Clinical Trial Service Unit and Epidemiological Studies Unit, Nuffield Department of Clinical Medicine, University of Oxford, Oxford OX2 6HE, United Kingdom; * address correspondence to this author at: Clinical Trial Service Unit and Epidemiological Studies Unit, Harkness Building, Radcliffe Infirmary, Oxford OX2 6HE, United Kingdom; fax 44-1865-558817, e-mail sarah.clark@ctsu.ox.ac.uk)

Increased blood homocysteine is a potentially modifiable risk factor for cardiovascular disease. In a recent meta-analysis of individual participant data from prospective epidemiologic studies, a 25% lower homocysteine concentration was associated with an 11% lower risk for ischemic heart disease and a 19% lower risk for stroke (1). Blood homocysteine is easily lowered by folic acid supplementation, and several large-scale randomized trials are currently underway to assess the effects of homocysteine-lowering vitamin supplements on the risk of vascular disease. If such trials demonstrate benefit, there will be increasing interest in homocysteine determinations to assess vascular disease risk. In addition, further large-scale epidemiologic studies may be required to investigate the association between homocysteine and cardiovascular disease in a wider range of populations. These would be facilitated by simple and cost-effective methods for blood collection and analysis.

One of the chief constraints in homocysteine measurements is the continuing production and release of homocysteine by red blood cells after venipuncture, which causes an artificial increase in plasma concentration of ~10% per hour (2, 3). It has been recommended, therefore, that blood samples for homocysteine measurements be drawn into tubes containing EDTA, chilled, or placed on ice immediately after collection and that the plasma be separated from the red cells within 1 h. Such procedures can be difficult to implement in large-scale epidemiologic studies or other situations in which samples have to be collected remotely (e.g., in multiple clinics or in people’s homes) and transported to a central laboratory. Use of NaF or acidic citrate has been advocated for stabilization of homocysteine in whole blood at ambient temperature for several hours before plasma separation, but there are few data available on longer-term stability (4, 5). Using commercially available Vacutainers, we evaluated the stability of homocysteine in whole blood over several days, using EDTA with or without NaF as preservative and with storage at room temperature or chilled conditions.

Participants in the study were healthy volunteers (two males and eight females; age range, 25–60 years) from our department who consented to have their blood drawn. No ethics committee approval was gained for this study. We collected blood from each of the volunteers into two 5-mL Vacutainers containing EDTA and two 7-mL Vacutainers containing disodium EDTA (7.0 mg) and NaF (17.5 mg; EDTA + NaF; final NaF concentration, 60 mmol/L; Becton Dickinson UK Limited). The Vacutainers were centrifuged immediately (2100g for 15 min at 4 °C), and a 300-μL aliquot of plasma was taken from each one and stored at −80 °C. The Vacutainers were then gently mixed to recombine the cells with the remaining plasma. For each individual’s samples, one EDTA and one EDTA + NaF Vacutainer were stored at room temperature (21 °C) and the other EDTA and EDTA + NaF Vacutainers were stored under chilled conditions (4 °C). At 1, 2, 3, 4, and 7 days after collection, the Vacutainers were recentrifuged, a 300-μL aliquot of plasma was taken and stored at −80 °C, and the Vacutainers were mixed and replaced. The plasma aliquots corresponding to each Vacutainer type, temperature condition, and time point for each volunteer were analyzed together in one analytical run to avoid run-to-run variability.

Before analysis by HPLC, plasma samples underwent chemical reduction of the sulfur bonds by the addition of tris(2-carboxy-ethyl)phosphine (TCEP), protein precipitation with trichloroacetic acid, and reaction with 4-aminosulfonyl-7-fluorozeno-2-oxa-1,3-diazole (ABD-F) to form fluorescent adducts. We injected 20 μL of the resulting extract onto a C<sub>18</sub> column (Bio-Rad Laboratories Ltd.) on a Waters Alliance chromatography system (Model 2690; Waters Ltd.). The system was equipped with a Waters scanning fluorescence detector (Model 474) operating at an excitation wavelength of 385 nm and an emission wavelength of 515 nm. Data collection, integration, and quantification were performed by the Waters Millennium 32 software. Within-batch CVs were <2% at homocysteine concentrations of 12.8, 22.5, and 31.9.