Measurements of acylcarnitines in plasma have several diagnostic applications, and various tandem mass spectrometry methods are more specific and accurate than earlier methods. Libert et al. (5) reported that propionylcarnitine (PrCRN) and carnitine (CRN) esters of 2-methyl-branched fatty acids with chain lengths of four to nine carbons were found in the urine of patients with propionic and methylmalonic acidemia. Chace et al. (6) reported results for a high-throughput method for PrCRN analysis from dried-blood spots for the diagnosis of propionic and methylmalonic acidemias in newborns. Increased concentrations of methylmalonic acid (MMA) in urine or serum are also regarded as reliable indicators of functional vitamin B12 deficiency (7–9). The relationship between the concentrations of MMA and CRN esters in patients with increased MMA attributable to vitamin B12 deficiency has not been established. Because mass spectrometric testing is useful in newborn screening (10), with analysis of acylcarnitines, and specifically propionylcarnitine, to identify patients with methylmalonic acidemia, we evaluated the utility of acylcarnitine analysis in patients with low vitamin B12 concentrations.

MMA was analyzed on a tandem mass spectrometer as described previously (11). Acylcarnitines were analyzed as follows. Aliquots (150 μL) of calibrators, controls, and patient sera were transferred into microcentrifuge tubes. Acetonitrile was added to each tube to bring the total volume to 650 μL. The tubes were vortex-mixed vigorously for 2 min and centrifuged at 10,000 g for 2 min; 50 μL of the organic phase was then transferred into an autosampler vial containing 50 μL of internal standard (IS) solution, containing d9-CRN, d9-acylcarnitine (d9-AcCRN), and d9-PrCRN. The d9-CRN was the IS for free CRN, d9-AcCRN was IS for AcCRN, and d9-PrCRN was the IS for PrCRN and the indistinguishable isomers, methylmalonylcarnitine and succinylcarnitine (C4-dicarbCRN).

A PE series 200 HPLC system (Perkin-Elmer Analytical Instruments) in conjunction with an API 3000 ( Applied Biosystems/ MDS SCIEX) tandem mass spectrometer was used for the acylcarnitine analysis. The analysis was performed by flow injection in a positive-ion mode with a Turbolonspray™ interface and mobile phase consisting of methanol and 0.2 g/L formic acid in water (90:10 by volume). Q1 and Q3 quadrupoles were optimized for free CRN, AcCRN, and PrCRN. The collision gas was nitrogen with cell pressure of 1.1 Pa. The TIS capillary voltage was 5.2 kV, the orifice voltage was 30 V, and the collision energy was 28 V. The mobile phase flow rate was 60 μL/min, and the injection volume was 15 μL. The testing was performed in the multiple reaction monitoring (MRM) mode. The MRM transitions used in the analysis were from the molecular ions of m/z 162, 204, 218, 262, 171, 207, and 221 for CRN, AcCRN, PrCRN, C4-dicarbCRN, d4-CRN, d7-AcCRN, and d7-PrCRN, respectively, to the same product ion of m/z 85 for all analytes. Patient samples were analyzed for vitamin B12 and creatinine within 3 days of collection and stored for 1–3 weeks at −70°C before analysis for acylcarnitines and MMA. CRN, AcCRN, and PrCRN solutions were prepared at a concentration of 250 nmol/L in acetonitrile. The calibrators were prepared at concentrations of 0.5, 1, 5, and 10 μmol/L in dialyzed serum free of acylcarnitines and MMA. The calibration curves for the MRM method were linear up to 100 μmol/L for all acylcarnitines. Imprecision was determined by triplicate analyses of samples supplemented with acylcarnitine calibrators at concentrations ranging from 1 to 10 μmol/L. The imprecision (CV) of the MRM method at all concentrations studied was <8.0%. The limit of detection was defined as the concentration of an analyte that produced a peak height five times greater than the baseline noise. The limits of detection for the MRM mode were 0.2, 0.1, and 0.05 μmol/L for CRN, AcCRN, and PrCRN, respectively. Vitamin B12 concentrations were determined on an Advia Centaur analyzer (Bayer Diagnostics) by a method based on competitive displacement.

A set of 51 serum samples from adults (18 men and 33 women) with low serum vitamin B12 concentrations (<150 pmol/L), and normal renal function (serum creatinine <124 μmol/L) was selected for assessing the relationship between MMA and acylcarnitines. All studies with samples from human subjects were approved by the Institutional Review Board of the University of Utah. The mean, median, and SD for patient age were 68, 74, and 17 years. MMA concentrations >0.4 μmol/L were considered to indicate possible vitamin B12 deficiency. Among the samples analyzed, 18 (35%) had MMA concentrations within reference values (<0.4 μmol/L), 23 had moderately increased MMA concentrations (0.4–2 μmol/L), and 10 had markedly increased MMA concentrations (>2 μmol/L). A control group of 37 samples from healthy controls was also analyzed for vitamin B12, MMA, and acylcarnitines (Table 1). Initially the samples were screened for acylcarnitines in a precursor ion mode. Evaluation of the results was performed for qualitative identification of >30 acylcar-
Acylcarnitines with molecular mass <450 Da. No acylcarnitines other than AcCRN, PrCRN, and C4-dicarbCRN were detected in any sample. Quantitative analyses of AcCRN, PrCRN, and C4-dicarbCRN were performed in the MRM mode. PrCRN was detected in all the analyzed samples. The nonparametric reference interval (2.5–97.5 percentiles) for PrCRN in adults was 0.14–0.54 μmol/L, based on the values obtained for the control group. The median and ranges for vitamin B12, CRN, AcCRN, PrCRN, and the ratios PrCRN:AcCRN and PrCRN:CRN for the different groups are presented in Table 1. The range of concentrations of all the analytes, except vitamin B12, overlapped between the groups. However, the PrCRN concentrations and PrCRN:AcCRN and PrCRN:CRN ratios for individuals with markedly increased MMA were up to 17-fold higher than the highest result in the control group. The PrCRN concentrations and the PrCRN:AcCRN ratio in the control group overlapped with those observed in patients with inborn errors, although the median values in patients with inborn errors were higher than the values that we observed (6).

**Table 1. Summary of quantitative results for the controls and patients with various degrees of vitamin B12 deficiency.**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>37</td>
<td>0.12 (0.10–0.26)</td>
</tr>
<tr>
<td>Low B12, normal MMA (&lt;0.4 μmol/L)</td>
<td>18</td>
<td>0.18 (0.08–0.32)</td>
</tr>
<tr>
<td>Low B12, moderately increased MMA (0.4–2 μmol/L)</td>
<td>23</td>
<td>0.64 (0.40–1.88)</td>
</tr>
<tr>
<td>Low B12, markedly increased MMA (&gt;2 μmol/L)</td>
<td>10</td>
<td>9.51 (2.13–54.40)</td>
</tr>
</tbody>
</table>

**Fig. 1.** Relationship between serum MMA and PrCRN for individuals with low vitamin B12 (r = 0.96; F = 546; n = 51; A) and for samples with MMA <2 μmol/L (r = 0.54; F = 16; n = 41; B).
We also evaluated the correlation between MMA and PrCRN concentrations (Fig. 1), and PrCRN:CRN and PrCRN:AcCRN ratios in the samples with low vitamin B₁₂. The results were evaluated using the Fisher test, which confirmed the statistical significance of the linear regression. Correlation coefficients (r) and Fisher test (F) values for the relationship between MMA and PrCRN, the PrCRN:CRN ratio, and the PrCRN:AcCRN ratio were as follows: r = 0.96, F = 546; r = 0.98, F = 954; and r = 0.95, F = 434, respectively. A poor correlation between MMA and PrCRN:CRN was observed for samples with MMA concentrations <2 µmol/L (r = 0.558; F = 17.6). No correlation was observed between the MMA and C₄-dicarbCRN concentrations in all groups (data not shown).

In summary, the only abnormal acylcarnitine detected in samples from patients with increased MMA was PrCRN. The range of PrCRN concentrations observed in the serum of healthy individuals was 0.14–0.54 µmol/L. Correlations were observed between MMA and PrCRN concentrations and between MMA and both PrCRN: AcCRN and PrCRN:CRN ratios only for samples with MMA >2 µmol/L. A weak correlation was found between PrCRN and MMA concentrations for samples with MMA <2 µmol/L. This study suggests that PrCRN may be increased in severe vitamin B₁₂ deficiency but cannot reliably detect early stages of this disorder. Notably, 35% of individuals with low serum vitamin B₁₂ concentrations had MMA concentrations in the reference interval, consistent with previous observations (12).

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References

Dried-Serum Spot Assay for Folate, Sean O’Broin* and Elaine Gutter† (1 Department of Haematology, St James’s Hospital, Dublin 8, Ireland; 2 Centers for Disease Control and Prevention, Atlanta, GA 30333; * address correspondence to this author at: Department of Haematology, St James’s Hospital, Dublin 8, Ireland; fax 353-1-4162012, e-mail nutrition@stjames.ie)

Recent interest in the folate status of diverse populations as a serious clinical and public health issue has been associated with substantial demands for blood folate analyses. Folate analysis by microbiological assays fulfills two important requirements for such screening by providing an acceptable detection limit and specificity at a low reagent cost. Dried blood spots (DBS) on special filter paper have been notably cost-effective as a blood sample collection device in epidemiologic field studies, allowing fingerstick peripheral blood sampling and ease of specimen transport and storage. We recently combined the advantages of a microbiological assay and DBS in an assay for erythrocyte folate (1). We now describe a dried-serum spot (DSS) assay for serum folate (SF).

Filter-paper cards, each with 15 preprinted circles, were used (Type 903; Schleicher & Schuell). Individual fresh serum or EDTA-plasma samples from clinic patients or healthy donor volunteers were used throughout. DSS were prepared by pipetting 50–150 µL of serum onto the paper and drying the papers on a rack for at least 5 h at room temperature before storage at −80 °C in resealable plastic bags with desiccant sachets (1). Some paper was preimpregnated with ascorbic acid in an effort to extend DSS folate stability. These 15 × 10-cm paper cards were immersed flat in 10 mL of 10 g/L ascorbic acid for 1 min, followed by air-drying on sheets of aluminum foil.

Whole DSS folates were analyzed after the entire serum spot (50 µL) was cut out using scissors. The spots were cut in half and placed in the base of a borosilicate tube (12 × 75 mm), covered with 2 mL of 5 g/L sodium ascorbate containing 0.5 mL/L Tween 80, mixed on a vortex-mixer, and sonicated for 15 min (Deacon Ultrasonics Ltd). The DSS eluate was considered to represent a 1:40 dilution of serum plus 1900 L of serum and was analyzed by microbiological assay (2) with Lactobacillus casei subsp. Rhamnosus (NCIMB 10463). DSS punches (6.35 mm) were considered to represent 10 µL of serum (or plasma) for the purpose of comparison and were analyzed after extraction at a ratio of 1:40 as above.

We usually analyze SF after diluting sera 1:20 (100 µL of serum plus 1900 µL of diluent) (2). Increased assay sensi-