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).

We thank MDS SCIEX for providing a LC-MS/MS instrument for preliminary studies; we also thank Drs. Arnecke and Koscher (LABOR, Munich, Germany) for providing deuterated acylcarnitines for use as internal standards.

References


Dried-Serum Spot Assay for Folate, Sean O’Broin1 and Elaine Gunter2 (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 DSS 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 and was analyzed by microbiological assay (2) with Lactobacillus casei subsp. Rhamnosus (NCIMB 10463).

DSS punches (6.5 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-
tivity was achieved by adding reconstituted assay medium (folic acid casei medium; Difco) to microtiter plate wells (300-µL; capacity) at one-half the usual volume (i.e., 100 µL instead of 200 µL) but twice the usual strength. This dilution permits the addition of 200 and 100 µL of DSS extracts, providing two dilutions. Our assay medium (100 mL) thus contained 14.1 g of Difco medium and 6 mg of chloramphenicol (Sigma) with 150 mg of ascorbic acid added per 100 mL of the cooled medium.

SF stability on paper was crucial, so we studied this on both plain and ascorbate-treated paper for 14 days at 4, 22, and 37 °C in the dark. DSS from 10 individual sera were stored in resealable plastic bags with desiccant under the various conditions, removed at days 1–7 and day 14, and stored at −80°C until being assayed. DSS folate concentrations were significantly less stable in plain paper (Fig. 1A) than in paper treated with ascorbic acid (Fig. 1B), with the most significant loss occurring during the first 12 h (which included the drying process).

DBS folate stability was also time and temperature dependent (1), but was greatly diminished on ascorbate-treated paper compared with plain paper. We attributed this to the fact that folate polyglutamates are more stable in the intact cell and that the crystallized ascorbate-impregnated paper actually contributed to erythrocyte lysis at the time of blood “spotting”, as well as to subsequent deconjugation of erythrocyte polyglutamates. Erythrocytes desiccated naturally, with limited lysis, on plain paper, affording excellent folate stability in the long term.

DSS have a large surface area:volume ratio, and SF instability in the artificial light in a typical laboratory setting has been noted (3). Initially, we observed no significant difference by paired t-test (P = 0.79) in the folate concentrations of DSS (n = 15) on plain paper when dried overnight either in the dark or on the laboratory bench under 40 W white fluorescent lights. A repeat stability experiment used both ascorbate-treated and plain paper. The control sera (n = 10) were stored at −20°C overnight after the addition of ascorbic acid (5 g/L), and their SF concentrations (8.2 ± 4 µg/L) and those of DSS on ascorbate-treated paper dried in the dark (8.14 ± 3.5 µg/L) or under light (8.14 ± 3.7 µg/L) were not significantly different, but the folates of DSS on plain paper dried in darkness (7.4 ± 3.1 µg/L) or under light (7.0 ± 2.5 µg/L) were significantly lower. We concluded that DSS for folate analysis should always be prepared on ascorbate-treated paper.

In certain successful screening programs, uniform DBS punches have been considered to represent precalibrated constant volumes (4). This approach obviates the need for volumetric accuracy when collecting blood samples under field conditions, but its suitability is contingent on the degree of analytical accuracy and reproducibility required in the analysis. DBS punches are successful in screening for phenylketonuria because phenylalanine concentrations are so high in positive cases; the same approach was not considered sufficiently accurate for analyses such as serum ferritins, which have precise clinical cutoff points that define deficiency (5). The potential of using DSS [or dried-plasma spot (DPS)] punches for folate analysis will depend on the uniformity of the serum or plasma distribution in the paper; however, this has not been studied.

We initially compared the volumes of punches from center and edge locations of both DSS and DPS after labeling with iodinated human serum albumin (125I-labeled HSA; Seralb-125; CIS bio International). Iodinated HSA was added at a rate of 50 µL/mL of serum or plasma, and DSS (or DPS) punch volumes were calculated by gamma counting and comparison with controls. Plasma was prepared from EDTA-anticoagulated blood. We attributed the significant difference (P < 0.001) between the calculated center punch (6.35 mm) volumes of DSS (3.85 ± 0.244 µL) and DPS (4.26 ± 0.344 µL) prepared from identical peripheral blood samples (n = 8) to the lower viscosity of serum in the absence of fibrin. Center punch volumes of 50-, 100-, and 150-µL DPS were also significantly different (P < 0.001), and the volumes of punches taken from the edges of DPS were significantly higher than those from their centers (P < 0.001).
We studied the distribution of folates in DSS, using a microbiological assay, and considered that a 6.35-mm punch represented 10 μL of serum for the purposes of calculation. We extracted and analyzed the folates of DSS (50 μL) center punches (n = 15) from each of two sera and noted reasonably consistent within-assay folate concentrations (CV <10%). We also studied the distribution of SF in paper, using 37 different donor sera over four separate experiments. DSS of different serum volumes (50, 100, and 150 μL) were prepared, and punches from either their edges or centers (or both) were analyzed. The folate concentrations were expressed as percentages of the control SF concentrations, and 50-μL DSS center (75.7% ± 17.5%), 150-μL DSS center (83.0% ± 16.5%), and 150-μL DSS edge punch (98.8% ± 22.9%) volumes were all significantly different. Center and edge punches from 100-μL DSS (extracted together) represented 82.5% ± 19.3% of the control.

Overall, the DSS folate and 125I-labeled HSA distributions were similar, being of significantly higher concentrations at DSS edges compared with their centers in all studies. In addition, the pretreatment of paper with ascorbic acid significantly limited the “spreadibility” of serum, producing smaller DSS with significantly increased punch volumes. We considered that the evenness of ascorbate distribution in paper might also affect SF distribution and concluded that fixed-size DSS punches were unsuitable for SF analysis because of these variables. We subsequently analyzed whole DSS (50 μL) on ascorbate-treated paper as described.

Folate assay performance was monitored using both conventional serum controls (2) and DSS controls. The assay had good reproducibility: for DSS from three different sera assayed 10 times each in a single assay, the (mean ± SD) results were 4.4 ± 0.3, 8.5 ± 0.6, and 9.05 ± 0.37 μg/L, respectively. Similar DSS controls for monitoring between-assay reproducibility were prepared by cutting the DSS from paper, placing the entire DSS in borosilicate tubes, capping the tubes with rubber stoppers, and storing them at −80 °C. On the day of an assay, a tube was removed, and the DSS was extracted with 2 mL of extraction solution as described. The between-assay reproducibility for three such DSS controls was good: in 10 separate assays over 12 months, the (mean ± SD) results were 4.8 ± 0.5, 9.7 ± 0.7, and 10.9 ± 1.0 μg/L, respectively. This indicates the potential of DSS as a vehicle for the long-term storage of SF assay control sera.

When we compared DSS folate concentrations (mean ± SD, 8.54 ± 4.45 μg/L) with SF concentrations analyzed conventionally (8.4 ± 4.6 μg/L) over several assays, they correlated well (DSS folate = 1.0SF − 0.1; r² = 0.95; n = 122), and were not significantly different by paired t-test (P = 0.2). Comparison of results in a difference plot (6) showed no correlation of between-method differences with concentration. The mean and SD of the differences were 0.024 and 1.06 μg/L, respectively.

DSS on ascorbate-treated paper offer a practical and economical storage vehicle for SF with potential for routine diagnostic use, for population-screening applications, and for storing control serum pools. The stability of DSS folate on ascorbate-treated paper (Fig. 1B) for up to 1 week at 20 °C and for 2 weeks at 4 °C indicates a potential for short-term storage in conventional refrigerators or for shipping by mail from collection sites.

References