Published in final edited form as:

J Pediatr. 2013 August ; 163(2): 596–597.e1. doi:10.1016/j.jpeds.2013.03.043.

Long-term stability of folate in dried blood spots stored in several conditions

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Abstract

We examined stability of folate in 50 subjects' dried cord blood spots stored for 9 months at –80 °C, 4 °C, ambient and humid conditions. Mean folate declined progressively, but most subjects were $\pm$ 3 ranks of their –80 °C position. Meaningful information about relative concentrations was retained across conditions.

Dried blood spots (DBS) remaining after newborn screening are stored by some departments of health (1) and offer a valuable resource for epidemiological studies. However, there is no standard for storage and the effects of varying storage conditions on analyte concentrations must be understood. Here we examined the stability of folate, which is essential for embryonic development, assayed in cord blood DBS from the same individuals stored at four different conditions for nine months.

Methods

We recruited healthy pregnant volunteers at the University of Minnesota Medical Center in Minneapolis, MN during 2008–2010; study procedures were approved by the University of Minnesota Institutional Review Board. Umbilical cord blood obtained from 158 newborns was spotted onto Whatman 903 filter paper. Four spots from each infant were dried, sealed, and stored for nine months with one at each condition: –80 °C, 4 °C, ambient (climate-controlled laboratory with 0–44% relative humidity and 24–27 °C), and humid (greenhouse with 53–68% relative humidity and 15–30 °C). DBS were then placed at –20 °C until assay. Matched DBS of the first 50 newborns were selected for analysis.

A 6.35mm punch, taken from the perimeter to minimize the effect of within-spot variability, was obtained from each DBS. Punches were reconstituted in 250 μ l of AATX solution by sonication (2). Each punch was assumed to represent 12.5 μ l of whole blood to determine concentrations (2).

Folate concentration was measured via microbiological assay using a whole-blood protocol (3). A standard plate using known folate concentrations was prepared with each assay, and the sample concentrations were determined according to the regression line. Each sample

²Supported by NIH T32 CA099936 and the Children's Cancer Research Fund, Minneapolis, MN

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Statements: There are no prior publications or submissions with any overlapping information, including studies and patients. This manuscript has not been and will not be submitted to another journal while under consideration at The Journal of Pediatrics. None of the authors has a conflict of interest to report. The main sponsor, the Children's Cancer Research Fund, Minneapolis, MN, had no role in study design, data collection, analysis, or publication. Rheanne K. Zimmerman wrote the first draft; no authors received payment to produce the manuscript. Each author has seen and approved this version of the manuscript.

was allotted four wells at two concentrations (1X and 2X). Samples were diluted with AATX solution so that readings fell within the range of the standard plate and sensitivity of this assay. The quality control samples and -80°C experimental samples were plated at total dilutions of 1:800 and 1:1600; 4°C samples at 1:400 and 1:800; and ambient and humid at 1:250 and 1:500. Assays were repeated if the coefficient of variation (CV) of the four wells of any sample was greater than 15% or if the result was $< 40\text{ nmol/L}$ (14% of samples). When two assays for a given sample had an acceptable CV%, the mean was used.

Hemoglobin concentration was measured using the same reconstituted sample using reagents from Sigma-Aldrich (2). Hematrol standards were used to make control wells and a Versamax microplate reader was used to find absorbance.

Hemoglobin-folate for each sample was calculated directly from assay results and erythrocyte folate estimated as follows (2):

$$RBC_{\text{folate}} \left(\frac{\text{nmol}}{\text{L}} \right) = \frac{\text{Hemoglobin Folate} \left(\frac{\text{nmol}}{\text{L}} \right) * \text{Hemoglobin} \left(\frac{\text{g}}{\text{L}} \right)}{\text{hematocrit}}$$

Generalized estimating equations (GEE) were used to generate 95% confidence intervals (CIs) for the mean concentrations of folate, hemoglobin, and hemoglobin-folate at each condition and for the slope of variation of the rank orders between conditions.

Data simulations assessed the effect of the observed level of exposure measurement error on point estimates in hypothetical studies. Continuous odds ratios (OR) were considered with the unit being 10 nmol/L change in whole blood folate concentration. The $50\text{--}80^{\circ}\text{C}$ folate values served as controls in a case-control study of 100 participants. Folate values for 50 case subjects were randomly generated four times to obtain ORs of 1.1, 1.2, 1.3, and 1.4, respectively. In each dataset the values were ranked, randomly shuffled between 0 and 3 places up or down (based on the observed range of rank variation), and then re-ranked. Each subject was reassigned a new folate value corresponding to the original value at their new rank. The OR was calculated for the new data set, and this procedure was repeated 1000 times for each of the four original ORs. The same simulation approach was used for hemoglobin-folate, with random shuffling between of rank 0 and 4 places up or down. All analyses were conducted using SAS version 9.2 (SAS Corporation, Cary, NC).

Results

Mean folate values decreased significantly with higher storage temperatures (Table 1). Hemoglobin was notably lower in the ambient and humid conditions than the other conditions, but absolute differences were small. Hemoglobin-folate values naturally show a combination of these trends, with substantial decreases between the -80°C , 4°C , ambient and humid conditions. The hemoglobin-folate point estimates reflect the trend noted for folate, the decline in values was less steep.

We graphed rank order of DBS in the four conditions (Figure 1 - online only) which by visual inspection showed that rank order was generally retained between storage conditions, with some mixing. GEE analysis of folate and hemoglobin rank order exhibited a slope CI of about -2.9 to 2.9 , meaning the data are consistent with a true slope between those values, while the hemoglobin-folate rank order slope CI was -3.9 to 3.9 .

Data simulations indicated that for ORs of 1.1 to 1.3 per 10 nmol/L increase in DBS folate the ORs after rank shuffling were very similar to the ORs in the original, unshuffled data

(Table 2). For instance, the mean OR of 1000 simulations of data with an original OR of 1.3 was 1.290. The difference between the mean OR of 1000 simulations, 1.299, and the original OR of 1.4 was notably greater; however, this degree of association would be unexpectedly large.

Discussion

We found that folate and hemoglobin concentration in DBS are substantially lowered by storage at warmer temperatures and greater humidity. Our study is thus consistent with prior research (2), but novel in the length of storage and diversity of conditions. We minimized variability by using matched DBS at each storage condition and uniformly obtaining punches from the DBS perimeter. In the folate assay, the average CV of the four wells for each sample was 9.3% while that for hemoglobin was 8.9%.

This study demonstrates that folate concentrations decrease with storage at warmer temperatures, as expected, thus folate concentrations cannot be meaningfully compared between individual samples that were stored at different conditions. Rank order is however largely retained among DBS stored at warmer temperatures, thus DBS stored under non-ideal conditions may be used for studies in which relative folate level is of interest.

Abbreviations and acronyms

DBS	dried blood spots
CV	coefficient of variation
GEE	generalized estimating equations

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Sample	-80°C	4°C	Ambient	Humid
1	1689	2656	1209	777
2	1730	1793	1381	1568
3	2143	1649	1676	1563
4	2164	1719	1076	537
5	2166	1908	1234	1189
6	2184	1696	1493	398
7	2425	1592	1358	1378
8	2512	1330	1646	1296
9	2515	1404	1691	1682
10	2535	1965	1389	366
11	2590	1348	1102	1323
12	2613	1883	1786	1462
13	2639	3472	2917	403
14	2640	2122	1287	1450
15	2647	2161	1913	599
16	2694	1641	1359	711
17	2731	2059	2154	1804
18	2735	1039	337	961
19	2740	2710	1946	1140
20	2775	1843	2439	1525
21	2858	2253	1732	940
22	2875	2156	1818	1769
23	2893	2114	1315	1310
24	2982	2484	1418	1450
25	2996	2111	1225	1171
26	3103	2042	1331	1256
27	3198	3038	2307	2293
28	3258	2405	1564	1312
29	3258	3192	2434	2009
30	3355	2267	1912	1800
31	3370	2579	2168	915
32	3373	2305	1448	1420
33	3426	3492	3394	2595
34	3441	2143	1448	1711
35	3609	1769	1464	1394
36	3641	2581	4286	2284
37	3691	2646	1482	1800
38	3925	2409	2361	2073
39	4103	1748	1173	883
40	4164	3478	2541	2343
41	4205	2738	2932	1444
42	4211	3468	1761	1916
43	4401	2751	1823	962
44	4670	2536	2226	1979
45	4728	2202	2528	1640
46	5272	2649	1916	1050
47	5525	5561	3838	3619
48	5975	3591	2992	2770
49	6133	1933	3179	1840
50	6199	2863	2333	3275

Figure 1.

Retention of rank order of hemoglobin-folate values (pmol/g) for samples stored at the four conditions. Each row represents one individual's samples, and the shade of gray in each box represents the rank within that condition.

Table 1

The folate, hemoglobin, and hemoglobin folate levels from DBS after storage for nine months at four conditions.

Folate (nmol/L)	−80 °C	4 °C	Ambient	Humid
Mean	384.1	263.4	167.0	147.5
Standard Deviation	135.9	100.9	65.3	75.2
95% CI	346.8, 421.4	241.8, 285	139.9, 194	122.1, 172.8
Hemoglobin (g/dL)	−80 °C	4 °C	Ambient	Humid
Mean	11.79	11.49	9.09	9.98
Standard Deviation	3.63	3.95	2.98	3.67
95% CI	10.8, 12.79	11.11, 11.86	8.56, 9.62	9.38, 10.59
Hemoglobin Folate (pmol/g)	−80 °C	4 °C	Ambient	Humid
Mean	3354	2350	1915	1507
Standard Deviation	1095	767	758	684
95% CI	3054, 3655	2089, 2610	1661, 2168	1268, 1746

Table 2

Simulation of odds ratios for DBS folate in a hypothetical case-control study

	Folate			Hemoglobin Folate		
	Simulated ORs (1000 replications of ± 3 rank shuffling)			Simulated ORs (1000 replications of ± 4 rank shuffling)		
Original OR	Mean	Min	Max	Mean	Min	Max
1.1	1.10	1.089	1.114	1.10	1.087	1.116
1.2	1.197	1.181	1.215	1.197	1.180	1.219
1.3	1.290	1.219	1.382	1.283	1.193	1.389
1.4	1.299	1.165	1.441	1.266	1.165	1.455