Clinical Chemistry 53:4
717–722 (2007)

Endocrinology and Metabolism

Long-Term Stability of Amino Acids and Acylcarnitines in Dried Blood Spots

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Background: **Dried blood filter cards, collected for newborn screening, are often stored for long periods of time. They may be suitable for the retrospective diagnosis of inborn errors of metabolism, but no data are currently available on the long-term stability of amino acids and acylcarnitine species.**

Methods: **We analyzed amino acids and acylcarnitines by tandem mass spectrometry in 660 anonymous, randomly selected filter cards from 1989 through 2004. We assessed long-term stability of metabolites by linear regression and estimated annual decrease of concentration for each metabolite.**

Results: **Concentrations of free carnitine increased by 7.6% per year during the first 5 years of storage and decreased by 1.4% per year thereafter. Alanine, arginine, leucine, methionine, and phenylalanine decreased by 6.5%, 3.3%, 3.1%, 7.3%, and 5.7% per year, respectively. Acetylcarnitine, propionylcarnitine, citrulline, glycine, and ornithine decreased by 18.5%, 27.4%, 8.1%, 14.7%, and 16.3% per year during the first 5 years, respectively; thereafter the decline was more gradual. Tyrosine decreased by 1.7% per year during the first 5 years and 7.9% per year thereafter. We could not analyze medium- and long-chain acylcarnitine species because of low physiological concentrations.**

Conclusions: **Estimation of the annual decrease of metabolites may allow for the retrospective diagnosis of inborn errors of metabolism in filter cards that have been stored for long periods of time.**

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Since the advent of newborn screening for phenylketonuria more than 35 years ago, neonatal screening programs have included many additional inborn errors of metabolism *(1, 2)*. In particular, electrospray ionization tandem mass spectrometry (ESI-MS/MS)[6] has added a number of different metabolites to the analytical repertoire. With ~100% sensitivity and specificity, ESI-MS/MS has become the predominant technique for neonatal screening of dried blood on filter cards for inborn errors of metabolism, including amino acidopathies, fatty acid oxidation disorders, and organic acidurias *(3)*.

Filter cards from each newborn infant are stored for up to 10 years as a source of valuable material for epidemiological studies and forensic purposes *(4)*. In infants who have died unexpectedly, retrospective diagnosis may be important to provide the family with genetic counseling *(5–12)*.

The diagnosis of inborn errors of metabolism by ESI-MS/MS is based on the quantitative analysis of amino acids and acylcarnitines *(2, 3)*. The concentrations of metabolites in affected infants exceed the respective population-dependent cutoff limits. In addition, metabolite concentrations depend on metabolic status, dietary intake *(2)*, and maturity of the infants *(13, 14)*, which may pose a diagnostic challenge. Time-dependent degradation of metabolites during long-term storage of dried blood filter cards may contribute to metabolite variability. To the best of our knowledge, however, few data are currently avail-

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Received August 3, 2006; accepted January 8, 2007.

Previously published online at DOI: 10.1373/clinchem.2006.076679

[6] Nonstandard abbreviations: ESI, electrospray ionization; MS/MS, tandem mass spectrometry.

able regarding the long-term stability of amino acids and acylcarnitines in dried blood filter cards.

Consequently, we decided to investigate the long-term stability of these metabolites in dried blood filter cards stored for >10 years. Our objectives were to identify simple mathematical models to describe the time-dependent change of metabolite concentrations while adjusting for random variation in subsequent years.

Materials and Methods

SAMPLES

We retrieved 60 dried blood filter cards, without conscious bias, from infants born in January of the years 1989, 1991, 1993, 1995, 1997, 1999, 2000, 2001, 2002, 2003, and 2004 (total n = 660) from the Neonatal Screening Archives in Prague, Czech Republic. We chose samples 1 at a time, not as a sequence of 60, to avoid sampling bias. Samples were handled appropriately before shipment by courier to the screening laboratory in Vienna.

Before June 2003, blood was collected between the 5th and 7th days of life; after that time, between the 3rd and 4th days of life. This minor change in infant age does not have any effect on metabolite concentrations *(15)*.

We stored the filter cards (Schleicher & Schuell 2992) at ambient temperature in a dry environment. The cards were sent from the respective birth clinics to the screening center through regular public mail in sealed and bagged envelopes; therefore, the effects of humidity, temperature, and light should be negligible. None of the filter cards was autoclaved.

ACYLCARNITINE AND AMINO ACID ANALYSIS

We analyzed all samples *(16)* within 3 weeks by the same tandem mass spectrometer (MS Wallac). We punched 3-mm discs (DBS Puncher, Wallac) from the center of the dried blood spots and transferred the discs to a 96-well microtiter plate (Greiner). Subsequently, we added 100 μL methanol extraction solution, mixed with stable isotope–deuterated internal standards (CIL). The concentrations of the internal standards were as follows: $^2H_9$-carnitine 0.8 μmol/L; $^2H_3$-acetylcarnitine 0.2 μmol/L; $^2H_3$-propionylcarnitine, $^2H_3$-butyrylcarnitine, $^2H_9$-isovalerylcarnitine, $^2H_3$-octanoylcarnitine, and $^2H_9$-myristoylcarnitine 0.04 μmol/L; $^2H_3$-palmitoylcarnitine 0.1 μmol/L; $^{15}N,2^{13}C$-Gly 12.5 μmol/L; and $^2H_4$-Ala, $^2H_8$-Val, $^2H_3$-Leu, $^2H_3$-Met, $^2H_5$-Phe, $^{13}C_6$-Tyr, $^2H_3$-Asp, $^2H_3$-Glu, $^2H_2$-Orn, $^2H_2$-Cit, and ^{13}C-Arg-HCl 2.5 μmol/L. The plate was sealed and mixed. The samples were centrifuged, and the eluate was evaporated under vacuum. We then incubated the samples with butanol and 10% acetylchloride. The resulting solution of butyl-esters of acylcarnitines and amino acids was again evaporated and subsequently reconstituted in 100 μL water:acetonitrile:formic acid (2500:2500:1). The source block temperature was 140 °C, nebulizer gas 160 L/h, and desolvation gas 518 L/h. We measured acylcarnitines by positive precursor ion scan of m/z 85 and amino acids by different neutral loss scan functions. Their concentrations were derived from the ratio of the ion intensity of the substance to the corresponding internal standard. We included low and high controls with each batch of samples (i.e., per 96-well plate). All samples and controls were analyzed in a single run sequence. A total of 7 runs were performed to analyze all 60 samples.

STATISTICAL ANALYSIS

Because the data were nongaussian in distribution, all data were described by median and interquartile range. Before further statistical computations, we transformed the concentrations of acylcarnitines and amino acids to a logarithmic scale. We assessed the effect of time of storage on the concentration of each metabolite by 2 linear regression models. In the 1st model, we assumed a constant decrease of concentration over the period of 15 years. This assumption may oversimplify the actual rate of change for some metabolites. Thus, in a 2nd model, we introduced a breakpoint after 5 years of storage, allowing for 2 different constant decreases before and after 5 years of storage. The breakpoint was not selected by means of statistical significance but rather was determined in advance such that there were equal amounts of data available before and after the breakpoint. Among all possible choices for the breakpoint, our choice statistically guaranteed the most precise results. To simplify interpretation of the results, we refrained from refining our regression models any further. We verified the adequacy of the 2nd model by testing the required additional regression parameter for statistical significance. The parameter estimates from the regression models could be interpreted as yearly declines in concentration, measured on the log scale. Thus, we obtained the annual percent decrease of concentration (L%) from the parameter estimates (B) by retransformation, $L\% = (\exp(B) - 1) \cdot 100\%$. The log transformation applied to the original concentrations ensured that the residuals (observed minus predicted values) were approximately normally distributed with equal variance. P values <0.05 were considered statistically significant. We used the SAS System v. 8.2 (SAS Institute Inc.) for statistical calculations.

Results

With the exception of free carnitine (C0) and Val, all metabolite concentrations showed a decrease (Table 1). C0 increased during the first 5 years of storage, with the largest increase occurring during the 1st year of storage, during which the concentration rose by ~40%. After storage for more than 5 years, C0 began to gradually decrease. Two different constant decreases/increases per year were assumed before and after 5 years of storage. Thus, we obtained 2 curves fitting the data by linear regression (Fig. 1). The concentration increased by 7.6% (95% CI, 5.8–9.3) per year during the first 5 years of storage and decreased by 1.4% (0.5–2.4) per year thereafter. Val may be regarded as stable, since no significant

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Table 1. Estimated change per year of free carnitine (C0), acetylcarnitine (C2), propionylcarnitine (C3), and amino acids during the first 5 years of storage (years of collection, 1999–2004) and between 5 and 15 years of storage (years of collection, 1989–1999).

Metabolite	Decrease [or increase] per year (1999–2004), $ (95% CI)	Decrease per year (1989–1999), % (95% CI)
Free carnitine (C0)	[7.6 (5.8–9.3)][a]	1.4 (0.4–2.3)
Acetylcarnitine (C2)	18.5 (16.4–20.6)	7.5 (6.5–8.4)
Propionylcarnitine (C3)	27.4 (23.7–31.2)	7.8 (6.2–9.4)
Valine	No change	No change
Alanine	6.5 (5.8–7.2)	6.5 (5.8–7.2)
Arginine	3.3 (2.9–3.7)	3.3 (2.9–3.7)
Leucine	3.1 (2.5–3.6)	3.2 (2.5–3.6)
Methionine	7.3 (6.7–8)	7.3 (6.7–8)
Phenylalanine	5.7 (5.0–6.3)	5.7 (5.0–6.3)
Citrulline	8.1 (6.5–9.7)	1.5 (0.8–2.3)
Glycine	14.7 (12.8–16.6)	4.4 (3.6–5.3)
Ornithine	16.3 (14.2–18.4)	3.5 (2.6–4.5)
Tyrosine	1.6 (−1.5–4.9)	7.9 (6.2–9.6)

[a] Increase rather than decrease.

change of concentration was found during the time period tested (Fig. 2 and Table 1).

The concentration changes for several amino acids, including Ala, Arg, Leu, Met, and Phe (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue4), may be described by a simple exponential model, as the estimated decrease per year was constant during the entire 15-year period (Table 1). The estimated percentile decrease per year for Ala, Arg, Leu, Met, and Phe was 6.5% (5.8–7.2), 3.3% (2.9–3.7), 3.1% (2.5–3.6), 7.3% (6.7–8.0), and 5.7% (5.0–6.3), respectively (Table 1).

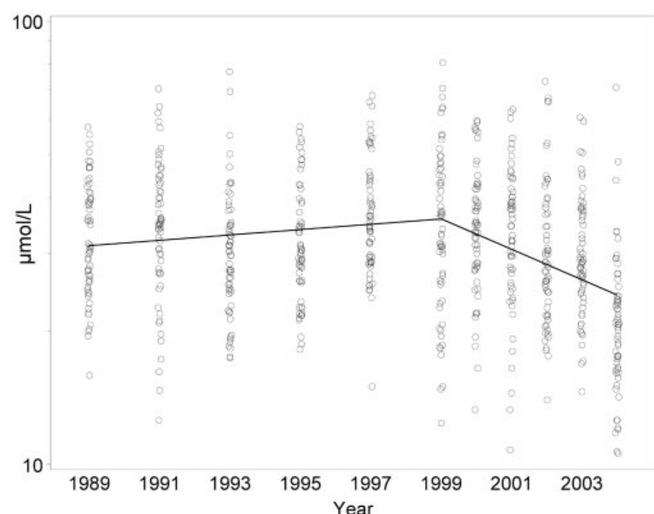

Fig. 1. Free carnitine.

Two straight lines fitting the data were obtained by linear regression. The x axis (logarithmic) represents the year of blood collection, the y axis the concentration in μmol/L.

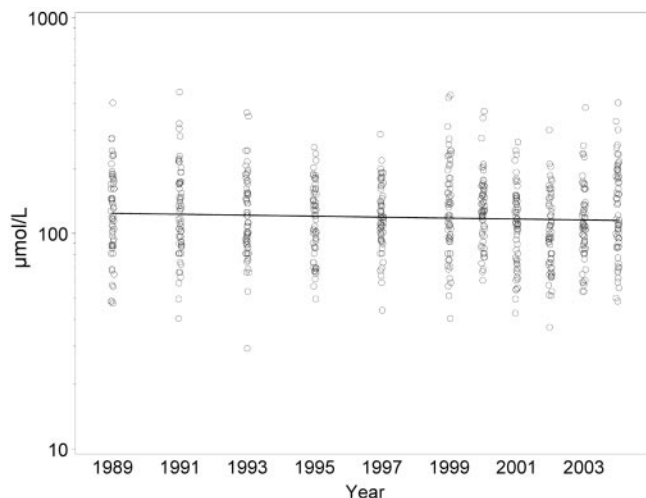

Fig. 2. Valine.

No significant change of Val concentration was observed. A horizontal line fitting the data was obtained by linear regression (see Fig. 1 in the online Data Supplement for phenylalanine). The x axis (logarithmic) represents the year of blood collection, the y axis the concentration in μmol/L.

The concentrations of C2, C3, Cit, Gly, and Orn decreased significantly during the 1st 5 years of storage and more gradually thereafter (Table 1, Fig. 3, and Fig. 2 in the online Data Supplement). The absolute values for all concentrations are also shown in a table in the online Data Supplement.

In contrast, the concentration of tyrosine was relatively stable, with an average annual decrease of 1.7% (−1.5 to 4.9) during the first 5 years of storage. After >5 years of storage, the estimated annual decrease was 7.9% (6.2–9.6).

Most acylcarnitines, including hexanoylcarnitine (C6), octanoylcarnitine (C8), and long-chain acylcarnitines, had to be excluded from our study, because their physiologic

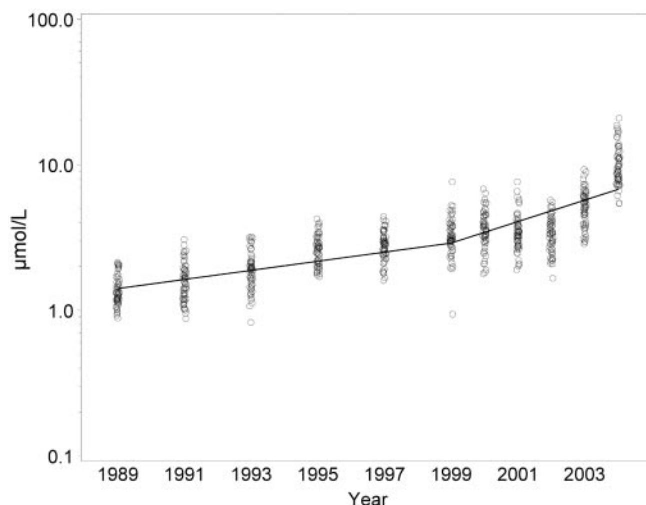

Fig. 3. Acetylcarnitine.

Two straight lines fitting the acquired data were obtained by linear regression (see Fig. 2 in the online Data Supplement for propionylcarnitine). The x axis (logarithmic) represents the year of blood collection, the y axis the concentration in μmol/L.

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concentrations are just above the detection limit of the ESI-MS/MS. We also had to eliminate Asp and Glu from the study because their mass spectra overlap when analyzed by MS/MS.

Discussion

Residual dried blood spot samples remaining after newborn screening constitute an important resource for health professionals, scientists, and epidemiologists *(17)*. Data on metabolite long-term stability would be valuable for retrospective diagnosis of inborn errors of metabolism to aid in genetic counseling of affected families *(5)*. We investigated amino acid and acylcarnitine concentrations, using ESI-MS/MS, in dried blood filter cards stored for up to 15 years.

A number of studies have examined the stability of genomic or viral DNA, thyroid-stimulating hormone, 17-α progesterone, or vitamin A *(18–21)*. Although these studies used a similar approach to investigate long-term stability, the biochemical properties of the investigated compounds are inherently different from acylcarnitine species and/or amino acids in dried blood spots. Consequently the results cannot be compared with those of our study.

Interestingly, only free carnitine concentrations showed an increase after storage, due to hydrolysis of esterified acylcarnitines *(22, 23)*. Whereas we were able to demonstrate an ~40% increase in free carnitine after 1 year of storage, other investigators observed, on average, a 20% increase during the same time period *(22, 23)*. The difference may result from different storage conditions of the filter cards. After 5 years of storage, concentrations of free carnitine gradually declined, likely because of reduced availability of the hydrolyzable esterified acylcarnitines. Short-chain acylcarnitine species (C2, C3) followed a different pattern than free carnitine, as hydrolysis continued (Table 1). The concentration of C3, and the ratios of C3/C2 and C3/C0, are important for the diagnosis of propionic aciduria or methylmalonic aciduria. The cutoff value for C3 in the Austrian screening laboratory is currently set at 3.5 μmol/L. Concentrations observed in infants with propionic aciduria/methylmalonic aciduria typically exceed 10 μmol/L *(2)*. Accounting for a mean decrease of 27.4% per year during the first 5 years, a retrospective diagnosis of propionic aciduria is still feasible after 3 years of storage. Values observed in some forms of methylmalonic aciduria, or defects of intracellular cobalamin metabolism, range from 6 to 10 μmol/L *(2)*, indicating that concentrations in samples older than 2 years may not be flagged as abnormal without correction for storage time.

Because of their low physiologic concentrations, most medium and long acylcarnitine species, as possible indicators for fatty acid oxidation defects, could not be evaluated in approximately two thirds of the neonatal screening population *(24)*. Alternatively, the stability of medium- and/or long-chain acylcarnitine species can be evaluated using artificially enriched dried blood spots. The stability of C6 and C8 acylcarnitine species, using blood spots enriched with C6 and C8 and stored in sealed plastic bags at 4 °C, has previously been established. After 4.5 years, the concentration decreases of C6 and C8 in blood were 17% and 15%, respectively *(22)*. Retrospective analysis of metabolites that are increased in inborn errors of metabolism would also allow evaluation of their stability in dried blood spots following prolonged storage. Dried blood filter cards from children with malonic aciduria were reanalyzed at different time intervals for up to 900 days, and malonylcarnitine was found to decrease exponentially, with a half-life of 248 days *(23)*.

Madira et al. *(24)* demonstrated a 50% to 60% decrease of phenylalanine during the first 5 years of storage at room temperature, although according to their graph, a decrease of ~20% occurred during the first 4 years, which is similar to our estimate of ~25% decrease after the same storage time. It is difficult to conceive why there should be a decrease of 30% during year 5. The article did not report the actual number of samples tested, nor whether samples had been autoclaved. To our knowledge no other studies addressing the issue of long-term stability of metabolites have been published.

According to our findings, Phe, Ala, Arg, Leu, and Met decrease exponentially, with a constant decrease during 15 years of storage (Table 1 and the table in the online Data Supplement). Methionine is the least stable of these amino acids, which corresponds to the previous report of Chace et al. *(25)*. These authors investigated the stability of amino acids in dried blood filter cards at 37 °C and found that the initial concentrations of Phe, Leu, Tyr, and Val decreased by 15% to 17%, whereas that of Met decreased by 24% after 30 days of storage *(25)*. More detailed information on concentration changes during storage was not provided *(25)*.

No significant decrease of Val was observed during the observation period, whereas the concentrations of Leu/Ile fell by an estimated 3.1% per year. At this rate of decrease, the initial concentration would have to be at least 630 μmol/L to exceed the 400 μmol/L cutoff after 15 years of storage. Because the observed concentration range for samples from children with maple syrup urine disease (319 to 3650 μmol/L) overlaps with the normal values, false-negative results may be obtained during neonatal screening, but in the majority of cases a retrospective diagnosis would still be feasible, even after 15 years of storage *(26, 27)*.

The concentration of citrulline decreased by ~8.1% per year during the first 5 years and by 1.5% per year thereafter. Consequently, the initial concentration should exceed 103 μmol/L to exceed the 60 μmol/L cutoff value after 15 years of storage. These values may be observed in infants with classic citrullinemia, which would most likely be detectable after prolonged storage without correction for storage time.

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Other factors may affect the results obtained from testing of dried blood spot filter cards. Limited freezer capacity and other constraints may prevent screening centers from storage of filter cards at lower temperatures. The concentrations of most amino acids and acylcarnitines vary considerably among the normal newborn population, depending on dietary intake, time of blood collection, hematocrit, and position of the punch within the dry blood spot *(2, 28)*. The concentration of some metabolites may be affected by overlapping spectra with other metabolites *(2)*. Last, the concentration of free carnitine may be unpredictably affected by hydrolysis of acylcarnitines during sample preparation *(22)*.

This study was initiated by the Middle European Society for Pediatric Endocrinology (MESPE) study group. The study material (newborn screening cards) was provided by the Czech Neonatal Screening Archive in Prague within the framework of the research project MSM 0021620814; laboratory facilities were provided by the Biochemical Genetics and National Neonatal Screening Laboratories in Vienna, Austria. K.A.S. was a recipient of financial support from PRO INFANTIBUS.

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