PEDIATRICS INTERNATIONAL Official Journal of the Japan Pediatric Society

Pediatrics International (2022) **64**, e15072 doi: 10.1111/ped.15072

Original Article

Stability of amino acids, free and acyl-carnitine in stored dried blood spots

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Abstract ***Background*:** Newborn screening of inborn errors of metabolism using tandem mass spectrometry has become a public health strategy in many developed countries. Retrospective analyses using stored dried blood specimens have been limited, mainly due to a lack of biochemical information on the long-term stability of acylcarnitines and amino acids in stored specimens. We studied the characteristic profiles of the stability of amino acid, free carnitine, and acyl carnitines in dried blood specimens stored in a refrigerator after newborn screening.

***Methods*:** Dried blood specimens from 198 healthy newborns, which had been stored in a refrigerator at 5 °C after newborn screening, were prospectively subjected to tandem mass spectrometry analyses after 1, 3, 6 months, 1 and 2 years of storage. We also retrospectively re-analyzed the stored samples from 90 newborns, which had been analyzed and stored at 5 °C for 4 years.

***Results*:** We found that proline (Pro) and tyrosine (Tyr) were stable for 2 years, and that alanine (Ala), arginine (Arg), and phenylalanine (Phe) decayed with linear regression. The C0 increased during the time-course of 2 years, whereas most acylcarnitines gradually decayed and some showed a linear correlation. The retrospective analysis of samples stored for 4 years revealed that Ala, Phe, Pro and Tyr were almost stable, leucine (Leu), valine (Val) decayed with linear regression, C0 increased, and C10, C12, C14, C14:1, C16, C18, C18:1 decreased, while maintaining a linear correlation.

***Conclusions*:** These data suggested that some metabolic parameters from refrigerator-stored dried blood specimens were applicable for the detection of inborn errors of metabolism.

Key words dried blood spot, inborn error of metabolism, newborn screening, sudden infant death syndrome, tandem mass spectrometry.

Inborn errors of metabolism (IEM) are a consequence of genetic defects that lead to a metabolic block in a biochemical pathway critical to tissue-specific cellular functions. Prompt and effective intervention in screening-positive patients is particularly important in cases of IEM.[1] Newborn mass screening has developed with the aim of identifying newborns who appear healthy but who could be at risk of developing metabolic attacks with the potential to lead to serious complications or death. Conventional technology using a bacterial inhibition assay, widely known as the Guthrie method, was developed around 1961 and has long been used. In the last decades, with the development of electrospray tandem mass-spectrometry (TMS), newborn screening to detect IEM by TMS has become a public health strategy in most developed and some developing countries.[2–7] This technology allows inexpensive, high-throughput, and simultaneous detection of approximately 30 different IEMs, which include most amino acid and organic acid disorders, and fatty acid oxidation defects using a single blood spot specimen.[8] Among them, rapid-onset disorders, such as fatty acid oxidation defects, organic acid metabolic disorders and urea cycle disorders, are critically important, otherwise these IEMs are diagnosed after the onset of life-threatening conditions.[9] The technology has been widely introduced in the USA and some European countries, and was introduced in Japan in 2014.[10]

Retrospective TMS using stored dried blood specimens (DBSs) would be applied effectively for individuals in whom newborn screening had already been performed by the Guthrie method alone, but who died for unknown reasons.[11,12] Nevertheless, it has been recognized that the results of retrospective TMS should be interpreted with caution because acylcarnitines are gradually hydrolyzed to increase free carnitine, and many amino acids are not stable in DBSs kept in hot and humid conditions.[13] DBSs may be appropriate for the retrospective diagnosis of IEMs if they are stored at −20 °C or −80°C.[14–16]

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Received 22 May 2021; revised 5 November 2021; accepted 19 November 2021.

However, the freezing capacity is limited in most laboratories and DBSs are conventionally stored in a laboratory refrigerator, and this is the most common situation in Japan. In addition, the guidelines for the clinical application of retrospective TMS have not been established, partly because of the lack of biochemical information on the long-term stability of acylcarnitines or amino acids in DBSs in the refrigerator.[17,18] Long-term stability was a topic of interest from 2000 to 2010 in the USA, EU and Japan, and many investigations were conducted by industrial companies during that period; however, publications have been limited, mainly because of the commercial basis of those studies. To our knowledge, there are only seven reliable articles concerning the long-term stability of DBS (Table 1).

In this study we investigated the characteristic profiles of the stability of each metabolite of amino acids, free carnitine, and acyl carnitines in DBSs stored in a refrigerator after the analysis by a non-TMS method at neonatal period. We aimed to develop calculation formulae by comparing the data obtained at newborn screening with the serially measured data of stored DBSs.

Methods

The prospective analysis over a 2 year period

In Oita Prefecture, DBSs were transferred by mail to the Department of Clinical Laboratory in Almeida Memorial Hospital, which was assigned as a laboratory center for newborn screening in Oita Prefecture. After the analysis, all the filter cards (Advantec Test Paper, PKU-S, Tokyo, Japan) were stored in no-sealed plastic bags, packed in cardboard boxes in the refrigerator room at 5 °C, 68–80% humidity. The stored DBSs from 198 babies born in July, 2018 were randomly picked up and were subjected to reanalyses of acylcarnitines and amino acids by TMS at 1 month, 3 months, 6 months, 1 year, and 2 years of storage. The non-derivatized TMS kit (Neobase Non-derivatized MSMS kit) from Perkin Elmer (Waltham, MA, USA) was used for the quantitative determination of amino acids and acylcarnitines in comparison with internal standards of stable isotope-labeled analytes. The measured metabolites consisted of 11 amino acids, free carnitine, and 21 acylcarnitines, which are applied as markers in screening for organic acidemia or fatty acid oxidation disorders. Acylcarnitines or amino acids in the DBS would degrade over time, so we developed a linear regression formula by comparing the data at newborn screening with the data at each re-evaluation of stored samples.

Retrospective analysis

After the analysis for the newborn screening test, DBSs were voluntarily stored at 5 °C in the Department of Clinical Laboratory in Almeida Memorial Hospital for approximately 10 years. We randomly selected stored DBSs from 90 newborns who were screened in July 2014. These DBSs were subjected to TMS again on August 2018, after 4 years of storage.

Table 1 Summary of the previous reports on the stability of metabolites in stored dried blood spots

Reference	Country	Samples	Sample Number	Storage temperature (humidity)	Storage period	Metabolic markers
Strnadova[15] (2007)	Australia	Newborns	60	Ambient temperature dry environment	15 years	AA, AC
Fingerhut[13] (2009)	Switzerland	N.D.	10	1.−18°C 2.18–25°C (25–60%)	1,000 days	AC
Adam[16] (2011)	USA	Human blood (after adjust of hematocrit)	36	1.37°C (<30%) 2.37°C (≥90%)	30 ± 5 days	TSH, T4, 17-OHP, IRT, T-GAL, GALT、BIOT, SUAC, AA, AC
Prentice[14] (2013)	UK	Newborns	N.D.	1.21°C (RT) 2.−20°C 3.−80°C	104 weeks	AA, AC
Golbahar[17] (2014)	Bahrain	Newborns	N.D.	1.RT 2.37°C (<30%) 3.45°C (<30%) 4.37°C (≥70%) 5.45°C (≥70%)	8 days	AA, AC
van Rijt[18] (2020)	Netherlands	Newborns	598	4°C (1st year) RT (after 2 year)	5 years	AC
The present study	Japan	newborns	198	5°C	2 years	AA, AC
The present study	Japan	newborns	90	5°C	4 years	AA, AC

AA, amino acid; AC, acylcarnitine; BIOT, biotinidase; T-GAL, total galactose; GALT, galactose-1-phosphate uridyltransferase; IRT, immunoreactive trypsinogen; N.D, not described; 17-OHP, 17α-hydroxyprogesterone; RT, room temperature; SUAC, succinylacetone; T4, thyroxine; TSH, thyroid-stimulating hormone.

Statistical analyses

Regression analysis was performed to the relationship between the data analyzed before storage and those analyzed after each storage interval (1 month, 3 months, 6 months, 1 year, 2 years). The data $R^2 > 0.5$ (i.e., $R > 0.7$ or $R < -0.7$) indicate positive or negative linear relationship.

Ethics

This study was approved by the ethics committee of Oita University Hospital, Oita, Japan (No. 1434).

Results

Prospective study for 2 years

The 198 DBSs analyzed for newborn screening and then stored at 5 °C were successfully reanalyzed for acylcarnitines and amino acids by TMS at the time-points of 1 month, 3 months, 6 months, 1 year, and 2 years of storage.

Amino acid data

The time-dependent changes in amino acid concentrations were evaluated over time, as shown (Table 2, Fig. S1). We found characteristic results, and classified the amino acids into four groups as follows: Group 1, almost stable for 2 years, proline (Pro) and tyrosine (Tyr); Group 2, decayed with linear regression ($R^2 \geq 0.5$), alanine (Ala), arginine (Arg), phenylalanine (Phe); Group 3, gradually decayed without linear regression, citrulline (Cit), leucine (Leu), valine (Val); Group 4, decayed promptly. glycine (Gly), methionine (Met), ornithine (Orn).

Carnitine data

The time-course changes and correlations of free carnitine and acyl-carnitines were evaluated (Table 3, Fig. S1). As shown in Table 4, C0 (free carnitine) increased during the time-course, whereas most acylcarnitines decayed or decreased during storage; C3, C5, C10, C14, C16, C18, C18:1 were almost stable, or decayed while maintaining a linear correlation with $R^2 \geq 0.5$ for 2 years whereas C2, C5:1, C5OH, C5DC, C8, C12, C14:1, C16OH, C18:1OH, were unstable during the storage. The ratios of C3/C2, C0/(C16 + C18), and C14/C3 gradually increased with keeping linear correlation with $R^2 \geq 0.5$.

Retrospective study of DBSs stored for 4 years

Ninety DBSs that were originally analyzed in July 2014 and which had been stored in a refrigerator at 5 °C were randomly selected, and then analyzed by TMS. As a result, we found that the Ala levels of some DBSs were remarkably low, regardless of the initial values at newborn screening. The storage in a refrigerator at 5 °C seemed insufficient to prevent bacterial or fungal contamination. Then, DBSs with Ala levels of <100 nmol/mL were excluded from the study because they were considered too low in relative to the initial values in the newborn period (12 samples; Fig. S2). We hypothesized that consumption by mold had occurred; although we did not analyze the DBSs for fungi.

Based on the analysis of 78 DBSs, the data of six amino acids, Ala, Arg, Leu, Orn, Phe, Pro, Tyr, and Val maintained a linear correlation for 4 years, most of these values showed a correlation for 2 years in the prospective study (Table 2, Fig. S1). The levels of C0, C5OH, C10, C12, C14, C14:1, C16, C18, C18:1 maintained a linear correlation for 4 years. The ratios of C0/(C16 + C18) also maintained a linear correlation, whereas those of C3/C2, (C16 + C18:1)/C2, C14:1/C2 C8/C10 and C14/C3 did not (Table 3, Fig. S1).

Discussion

Amino acids in blood samples except for Met are stable at ≤4 °C for 1 month and should be stored long-term at ≤-80 °C; however, storage at −20 °C is not recommended, as the enzymes in the plasma maintain their catalyzing reaction activity in a crystalline ice matrix interspersed with a dense liquid phase at −20 °C. In contrast, freezing of DBS at −20 °C has been shown to be sufficient for effective storage for at least 2 years. The initial drying process and resulting lack of a liquid matrix in DBS are proposed to be responsible for the stability of amino acids in DBS.[14] In the present study, we showed that DBSs stored at 5 °C for 4 years were able to be analyzed by TMS, and that some amino acids, free carnitine, and acylcarnitine might be applicable for retrospective detection of IEMs. Newborn screening tests using TMS remain unimplemented in many countries;[19–21] congenital hypothyroidism, which is detected by the measurement of TSH, is the only disease for which screening is performed in many developing countries.[2,3] The retrospective analysis of stored DBSs (stored at 5 °C and not under ideal conditions) by TMS would be beneficial for people in these countries.

The potential estimation of stored DBSs in the diagnosis of IEMs by TMS is shown in Table 4. Among the IEMs of amino acids, patients with prolinemia (Pro), tyrosinemia (Tyr), argininemia (Arg), and phenylketonuria (Phe) could be retrospectively identified by TMS using DBSs that had been stored for 4 years after appropriately applying a calculation formula. Using the data on acylcarnitines, systemic carnitine deficiency (C0),[22,23] isovaleric acidemia (C5), carnitine palmitoyl transferase 1 (CPT1) deficiency [C0/(C16+C18)][24,25] could be identified as organic acidemias and fatty acid oxidation disorders (FAODs). Methylmalonic acidemia (C3 and C3/C2), propionic acidemia (C3 and C3/C2), medium-chain acyl-CoA dehydrogenase deficiency (MCAD) (C8 and C8/C10) and carnitine acylcarnitine translocase deficiency (C16 and [C16 + C18:1]/C2) could be identified. However, patients with methylmalonic acidemia, propionic acidemia, or carnitine acylcarnitine translocase deficiency can be missed because of a higher false-positive rate due to lower C2 values. The C2

Table 2 The linear regression formulae and coefficient correlation of R^2 (amino acids)

	1 year (n = 198)				2 years (n = 198)	4 years (n = 78)
	1 month	3 months	6 months	1 year		
Ala	$R^2 = 0.75$ $y = 0.85x + 44.36$	$R^2 = 0.71$ $y = 0.93x + 48.89$	$R^2 = 0.57$ $y = 0.79x + 77.73$	$R^2 = 0.61$ $y = 0.68x + 77.85$	$R^2 = 0.61$ $y = 0.74x + 54.53$	$R^2 = 0.60$ $y = 0.90x + 27.61$
Arg	$R^2 = 0.82$ $y = 0.81x + 1.45$	$R^2 = 0.79$ $y = 0.87x + 1.14$	$R^2 = 0.75$ $y = 1.13x + 2.50$	$R^2 = 0.72$ $y = 1.38x + 1.91$	$R^2 = 0.76$ $y = 0.82x + 1.97$	$R^2 = 0.57$ $y = 0.48x - 0.05$
Cit	$R^2 = 0.56$ $y = 0.71x + 4,07$	$R^2 = 0.42$ $y = 0.66x + 5.65$	$R^2 = 0.55$ $y = 0.75x + 4.95$	$R^2 = 0.53$ $y = 0.63x + 5.86$	$R^2 = 0.46$ $y = 0.66x + 3.86$	$R^2 = 0.46$ $y = 0.93x + 2.20$
Gly	$R^2 = 0.60$ $y = 0.73x + 120.07$	$R^2 = 0.49$ $y = 0.77x + 129.93$	$R^2 = 0.21$ $y = 0.53x + 216.13$	$R^2 = 0.27$ $y = 0.43x + 185.04$	$R^2 = 0.34$ y = 0.55x + 124.7	$R^2 = 0.40$ $y = 0.56x + 95.06$
Leu	$R^2 = 0.68$ $y = 0.80x + 43.64$	$R^2 = 0.64$ $y = 0.82x + 52.24$	$R^2 = 0.33$ $y = 0.56x + 87.82$	$R^2 = 0.46$ $y = 0.62x + 61.83$	$R^2 = 0.51$ $y = 0.63x + 55.38$	$R^2 = 0.76$ $y = 0.94x + 24.46$
Met	$R^2 = 0.3$ $y = 0.63x + 8.61$	$R^2 = 0.55$ $y = 0.68x + 6.26$	$R^2 = 0.25$ $y = 0.39x + 8.50$	$R^2 = 0.23$ $y = 0.31x + 4.01$	$R^2 = 0.098$ $y = 0.13x + 0.99$	$R^2 = 0.01$ $y = 0.02x + 2.76$
Orn	$R^2 = 0.66$ $y = 0.57x + 27.49$	$R^2 = 0.51$ $y = 0.58x + 27.10$	$R^2 = 0.30$ $y = 0.73x + 52.28$	$R^2 = 0.36$ $y = 0.61x + 45.68$	$R^2 = 0.33$ $y = 0.43x + 23.75$	$R^2 = 0.55$ $y = 0.37x + 14.81$
Phe	$R^2 = 0.68$ $y = 0.77x + 10.46$	$R^2 = 0.66$ $y = 0.75x + 12.80$	$R^2 = 0.57$ $y = 0.79x + 13.45$	$R^2 = 0.57$ $y = 0.70x + 15.8$	$R^2 = 0.55$ $y = 0.61x + 15.09$	$R^2 = 0.70$ $y = 0.99x + 3.9$
Pro	$R^2 = 0.87$ $y = 0.97x + 12.04$	$R^2 = 0.86$ $y = 0.98x + 20.62$	$R^2 = 0.75$ $y = 0.98x + 40.22$	$R^2 = 0.82$ $y = 0.90x + 21.68$	$R^2 = 0.80$ $y = 1.00x + 12.73$	$R^2 = 0.74$ $y = 1.16x - 5.22$
Tyr	$R^2 = 0.95$ $y = 1.03x + 1.75$	$R^2 = 0.95$ $y = 1.10x + 1.66$	$R^2 = 0.92$ $y = 1.03x + 6.24$	$R^2 = 0.93$ $y = 1.03x + 6.22$	$R^2 = 0.93$ y = 0.94x + 5.41	$R^2 = 0.83$ $y = 1.06x + 14.48$
Val	$R^2 = 0.74$ $y = 0.79x + 26.10$	$R^2 = 0.68$ $y = 0.80x + 28.63$	$R^2 = 0.47$ $y = 0.76x + 54.84$	$R^2 = 0.60$ $y = 0.74x + 32.90$	$R^2 = 0.55$ y = 0.78x + 33.71	$R^2 = 0.66$ $y = 0.82x + 34.72$

The shaded columns indicate the items with a linear correlation with $R^2 \geq 0.5$. Ala, alanine; Arg, arginine; Cit, citrulline; Gly, glycine; Leu, leucine; Met, methionine; Orn, ornithine; Phe, phenylalanine; Pro, proline; Tyr, tyrosine; Val, valine.

Table 3 The linear regression formulae and coefficient correlation of R^2 (free carnitine and acylcarnitines)

	1year (n = 198)				2 years (n = 198)	4 years (n = 78)
	1month	3months	6months	1year		
C0	$R^2 = 0.86$ $y = 1.07x + 1.43$	$R^2 = 0.82$ $y = 1.15x + 3.10$	$R^2 = 0.75$ $y = 1.22x + 5.68$	$R^2 = 0.76$ $y = 1.35x + 6.56$	$R^2 = 0.71$ $y = 1.49x + 7.29$	$R^2 = 0.80$ $y = 2.29x + 5.44$
C2	$R^2 = 0.85$ $y = 0.85x + 0.23$	$R^2 = 0.79$ $y = 0.72x + 0.13$	$R^2 = 0.63$ $y = 0.51x + 0.63$	$R^2 = 0.56$ $y = 0.29x - 0.13$	$R^2 = 0.40$ $y = 0.15x - 0.27$	$R^2 = 0.06$ $y = 0.009x + 0.05$
C3	$R^2 = 0.89$ $y = 0.92x + 0.05$	$R^2 = 0.88$ $y = 0.89x + 0.03$	$R^2 = 0.80$ $y = 0.75x + 0.07$	$R^2 = 0.79$ $y = 0.56x - 0.01$	$R^2 = 0.66$ $y = 0.34x + 0.00$	$R^2 = 0.08$ $y = 0.06x + 0.06$
C5	$R^2 = 0.85$ $y = 1.02x + 0.01$	$R^2 = 0.83$ $y = 1.04x + 0.02$	$R^2 = 0.72$ $y = 1.09x + 0.02$	$R^2 = 0.77$ $y = 0.92x + 0.02$	$R^2 = 0.78$ $y = 1.02x + 0.00$	$R^2 = 0.45$ $y = 0.68x + 0.01$
C5:1	$R^2 = 0.01$ $y = 0.1x + 0.01$	$R^2 = 0.00$ $y = 0.01x + 0.01$	$R^2 = 0.00$ $y = 0.02x + 0.01$	$R^2 = 0.01$ $y = 0.09x + 0.01$	$R^2 = 5E\text{-}05$ $y = -0.008x + 0.01$	$R^2 = 0.01$ $y = 0.13x + 0.01$
C5OH	$R^2 = 0.79$ $y = 0.87x + 0.03$	$R^2 = 0.80$ $y = 0.84x + 0.03$	$R^2 = 0.60$ $y = 0.47x + 0.04$	$R^2 = 0.58$ $y = 0.48x + 0.04$	$R^2 = 0.42$ $y = 0.41x + 0.03$	$R^2 = 0.53$ $y = 0.40x + 0.03$
C5DC	$R^2 = 0.47$ $y = 0.71\ x + 0.03$	$R^2 = 0.44$ $y = 0.75\ x + 0.04$	$R^2 = 0.19$ $y = 0.46\ x + 0.05$	$R^2 = 0.20$ $y = 0.42\ x + 0.03$	$R^2 = 0.13$ $y = 0.35\ x + 0.02$	$R^2 = 9E\text{-}05$ $y = 0.003\ x + 0.03$
C8	$R^2 = 0.44$ $y = 0.76x + 0.02$	$R^2 = 0.53$ $y = 0.83x + 0.01$	$R^2 = 0.49$ $y = 0.72x + 0.02$	$R^2 = 0.30$ $y = 0.67x + 0.04$	$R^2 = 0.23$ $y = 0.63x + 0.04$	$R^2 = 0.38$ $y = 0.55x + 0.03$
C10	$R^2 = 0.73$ $y = 0.98x + 0.00$	$R^2 = 0.70$ $y = 0.99x + 0.01$	$R^2 = 0.68$ $y = 0.94x + 0.01$	$R^2 = 0.64$ $y = 0.88x + 0.01$	$R^2 = 0.59$ $y = 0.86x + 0.02$	$R^2 = 0.67$ $y = 0.72x + 0.001$
C12	$R^2 = 0.46$ $y = 0.73x + 0.03$	$R^2 = 0.52$ $y = 0.78x + 0.03$	$R^2 = 0.50$ $y = 0.74x + 0.03$	$R^2 = 0.48$ $y = 0.62x + 0.03$	$R^2 = 0.41$ $y = 0.62x + 0.03$	$R^2 = 0.77$ $y = 0.80x + 0.01$
C14	$R^2 = 0.81$ $y = 0.92x + 0.03$	$R^2 = 0.84$ $y = 1.03x + 0.03$	$R^2 = 0.60$ $y = 0.77x + 0.07$	$R^2 = 0.73$ $y = 0.75x + 0.05$	$R^2 = 0.70$ $y = 0.9x + 0.04$	$R^2 = 0.69$ $y = 1.01x + 0.02$
C14:1	$R^2 = 0.55$ $y = 0.86x + 0.02$	$R^2 = 0.54$ $y = 0.80x + 0.02$	$R^2 = 0.43$ $y = 0.83x + 0.05$	$R^2 = 0.25$ $y = 0.74x + 0.05$	$R^2 = 0.37$ $y = 0.93x + 0.06$	$R^2 = 0.55$ $y = 0.68x + 0.03$
C16	$R^2 = 0.85$ $y = 1.01x + 0.10$	$R^2 = 0.87$ $y = 1.07x + 0.17$	$R^2 = 0.81$ $y = 1.06x + 0.17$	$R^2 = 0.83$ $y = 0.97x + 0.20$	$R^2 = 0.81$ $y = 0.94x + 0.14$	$R^2 = 0.70$ $y = 0.91x + 0.37$
C16OH	$R^2 = 0.31$ $y = 0.64x + 0.01$	$R^2 = 0.18$ $y = 0.56x + 0.01$	$R^2 = 0.20$ $y = 0.60x + 0.01$	$R^2 = 0.19$ $y = 0.54x + 0.01$	$R^2 = 0.15$ $y = 0.43x + 0.00$	$R^2 = 0.47$ $y = 0.98x + 0.005$
C18	$R^2 = 0.83$ $y = 0.98x + 0.03$	$R^2 = 0.83$ $y = 1.00x + 0.06$	$R^2 = 0.82$ $y = 1.02x + 0.03$	$R^2 = 0.83$ $y = 0.91x + 0.06$	$R^2 = 0.77$ $y = 0.79x + 0.07$	$R^2 = 0.63$ $y = 0.86x + 0.16$
C18:1	$R^2 = 0.75$ $y = 0.90x + 0.12$	$R^2 = 0.73$ $y = 0.88x + 0.20$	$R^2 = 0.75$ $y = 1.03x + 0.10$	$R^2 = 0.76$ $y = 1.05\ x + 0.14$	$R^2 = 0.67$ $y = 0.85x + 0.17$	$R^2 = 0.62$ $y = 0.70x + 0.22$
C18:1OH	$R^2 = 0.24$ $y = 0.48x + 0.01$	$R^2 = 0.20$ $y = 0.44x + 0.01$	$R^2 = 0.21$ $y = 0.34x + 0.01$	$R^2 = 0.17$ $y = 0.36x + 0.01$	$R^2 = 0.09$ $y = 0.24x + 0.01$	$R^2 = 0.23$ $y = 0.85x + 0.01$
C3/C2	$R^2 = 0.97$ $y = 1.08x + 0.00$	$R^2 = 0.95$ $y = 1.22x + 0.00$	$R^2 = 0.92$ $y = 1.44x + 0.00$	$R^2 = 0.86$ $y = 2.04x - 0.01$	$R^2 = 0.69$ $y = 2.88x - 0.00$	$R^2 = 0.26$ $y = 7.48x + 0.19$
C0/(C16+C18)	$R^2 = 0.94$ $y = 1.05x + 0.32$	$R^2 = 0.92$ $y = 1.04x + 0.86$	$R^2 = 0.91$ $y = 1.19\ x + 1.21$	$R^2 = 0.90$ $y = 1.34\ x + 2.12$	$R^2 = 0.89$ $y = 1.54x + 2.71$	$R^2 = 0.78$ $y = 1.51\ x + 5.44$
(C16+C18:1)/C2	$R^2 = 0.93$ $y = 1.17x + 0.01$	$R^2 = 0.87$ $y = 1.50x + 0.01$	$R^2 = 0.72$ $y = 2.07x + 0.1$	$R^2 = 0.47$ $y = 3.99x + 0.03$	$R^2 = 0.25$ $y = 9.35\ x - 0.11$	$R^2 = 0.01$ $y = 78.04x + 14.75$
C14:1/C2	$R^2 = 0.59$ $y = 0.97x + 0.00$	$R^2 = 0.55$ $y = 1.22x + 0.00$	$R^2 = 0.36$ $y = 2.00x + 0.00$	$R^2 = 0.17$ $y = 4.18x + 0.01$	$R^2 = 0.19$ $y = 13.76x + 0.02$	$R^2 = 0.07$ $y = 196.72x - 0.01$
C8/C10	$R^2 = 0.11$ $y = 0.39x + 0.72$	$R^2 = 0.11$ $y = 0.33x + 0.75$	$R^2 = 0.15$ $y = 0.35x + 0.62$	$R^2 = 0.06$ $y = 0.45 + 0.88$	$R^2 = 0.05$ $y = 0.48\ x + 0.78$	$R^2 = 0.04$ $y = 0.53\ x + 0.73$
C14/C3	$R^2 = 0.92$ $y = 1.14x + 0.00$	$R^2 = 0.90$ $y = 1.26x + 0.01$	$R^2 = 0.87$ $y = 1.39x + 0.01$	$R^2 = 0.80$ $y = 1.88x + 0.00$	$R^2 = 0.62$ $y = 3.48 - 0.00$	$R^2 = 0.01$ $y = -33.00x + 8.41$

The shaded columns indicate the items maintaining a linear correlation with $R^2 \geq 0.5$.

Table 4 Diagnostic markers for inborn errors of metabolism are applicable to screening by tandem mass spectrometry using dried blood specimens

	Disease	Markers (value of cut-off)[†]
Organic acidemias	Methylmalonic acidemia/ Propionic acidemia	C3 (>3.6) C3/C2 (>0.22)
	Isovaleric acidemia	C5(>1.0)
	Methylcrotonylglycinuria	C5-OH (>1.0)
	Multiple carboxylase deficiency	C5-OH (>1.0)
	3-Hydroxy-3-methylglutaric acidemia	C5-OH (>1.0)
	Glutaric acidemia type 1	C5-DC (>0.20)
	β-Ketothiolase deficiency	C5-OH (>1.0) C5:1 (>0.025)
Fatty acid oxidation disorders	Medium-chain acyl-CoA dehydrogenase deficiency	C8 (>0.30) C8/C10 (>1.70)
	Very-long-chain acyl-CoA dehydrogenase deficiency	C14:1 (>0.25) C14:1/ C2 (>0.013)
	Trifunctional protein deficiency Long-chain acyl-CoA dehydrogenase deficiency	C16-OH (>0.08) C18:1-OH (>0.05)
	Carnitine palmitoyltranseferase 1 deficiency	C0/(C16+C18) (>40)
	Carnitine palmitoyltranseferase 2 deficiency	C14/C3 (>0.3) (C16 + C18:1)/C2 (>0.5)
	Carnitine acylcarnitine translocase deficiency	(C16 + 18:1)/C2 (>0.55) C16 (>5.0)
	Systemic carnitine deficiency	C0 (<7.5)
	Glutaric acidemia type 2	C8 (>0.3) C10 (>0.4) C12 (>0.4)

[†]The cut-off levels are set at the average value ±6 SD. These levels are applied for detection of the candidate diseases as the newborn screening in the Department of Clinical Laboratory in Almeida Memorial Hospital as following the previous report.[29]

level shows a marked decrease over the years; thus, the C3/C2 ratio should be carefully evaluated for the diagnosis of these disorders. Furthermore, patients with MCAD deficiency are also likely to be missed due to the reduced C8 value. The C0 data should be also interpreted with care for the diagnosis of systemic carnitine deficiency or CPT1 deficiency because the production of C0 (free carnitine) from acylcarnitine decay might greatly depend on the condition of the stored DBS. In a previous study, an infant with sudden infant death syndrome (SIDS) was biochemically and genetically diagnosed with MCAD deficiency based on a retrospective analysis of DBSs.[28] The C8 level was the key marker for MCAD deficiency and the level of C8 in the stored DBS of a deceased patient with MCAD deficiency was remarkably high. The data of the present study combined with the previous report suggested that MCAD might be detectable using stored DBS; however, the C8 level should be carefully assessed based on the results of the stored samples.

The clinical phenotypes of metabolic disorders with a slow onset, such as phenylketonuria (PKU)[26,27] or prolinemia, appear not only during infancy but also in childhood or adulthood. Thus, individuals with these rare IEMs, whose DBSs have not been analyzed by the Guthrie method or TMS, could be identified by the TMS using stored DBSs. Fresh plasma samples are undoubtedly desirable for the analysis of amino acids in blood, but it may be possible to perform TMS analysis using stored DBSs in developing countries and regions where TMS is not routinely performed for newborn screening. Furthermore, DBSs can be transferred to foreign laboratories under conditions of international cooperation during critical situations, such as natural disasters.

The present study was associated with some limitations. First, the cause of the time-independent degradation of metabolites in DBSs might include contamination, especially mold contamination, during long-term refrigerator storage. We found that the levels of most metabolites were extremely low in some DBS and that DBSs with low Ala levels could be differentiated from the others and were apparently contaminated by mold roughly estimated by visual or smell inspection (Fig. S2). We excluded these DBSs from the further analysis of metabolites. We think initial screening to select intact DBSs based on the Ala level would be practically reasonable; however, we did not subject DBSs to real-time polymerase chain reaction (PCR) or mold culturing. Further studies are needed to develop a protocol to properly select uncontaminated DBSs. Second, the DBSs of healthy newborns are said to comprise very low levels of medium- or long-chain acylcarnitines, such as C5, C5:1, C5OH, C5DC, C8, C10, C12, C14, C14:1, C16OH and C18:1OH. It might therefore be difficult to accurately estimate the decay of trivial carnitines during storage. Third, we did not analyze the samples of patients with IEMs whose bloodspots might include extremely high levels of metabolites. Final, this study was only a single-center analysis. Further multicenter analyses with DBS collection in different seasons and more samples are needed to establish application for the analysis of stored DBSs by TMS. These additional studies would also be important for developing guidelines for the clinical use of stored DBSs on a nationwide scale.

The targets of retrospective analyses using DBS have recently been expanded to include genetic analyses for intrauterine viral infections, human genome sequencing, or enzyme activities for inborn errors of metabolisms. Further studies on the analyte-specific stability of stored DBS will be important for expanding the potential applications of DBSs.

Conclusions

We demonstrated that, if appropriately stored in refrigerators, stored DBSs could be analyzed by TMS for the diagnosis of several IEMs. In the future, a genetic analysis using DBSs, with a focus on candidate genes detected by TMS, may help to raise the accuracy of the diagnosis of IEMs. Although the use of DBSs for research purposes is associated with ethical issues, the proper application of the new technology of TMS and gene testing would help to diagnose rare genetic diseases causing fetal sequelae and to provide accurate information for

the subsequent application of genetic counseling for the family.

Acknowledgments

We thank Teruko Suematsu (Department of Clinical Laboratory, Oita City Medical Association Almeida Memorial Hospital, Oita, Japan) for help in TMS analysis.

Funding information

The work was supported in part by JSPS KAKENHI Grant number 18K10465 (Y. Shimada) and the Bundai Researcher Team (BURST) grant number 15 (K. Ihara).

Disclosure

The authors declare no conflict of interest.

Author contributions

Y.S. and K.I. contributed to the study conception and design. N.K. was involved in the data collection and provided conceptual advice. M.G. and H.W. performed the analysis for MS/MS. Y.S. drafted the manuscript. K.I. and N.K. critically reviewed the manuscript and supervised the whole study process. All authors read and approved the final manuscript.

Informed consent

Informed consent was obtained in the form of an opt out on the website (https://www.med.oita-u.ac.jp/hospital/kenkyu-rinri/index.html).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Scatter plot with the linear regression formulae and coefficient of correlation (R^2). A, Amino acids. B, Free carnitine and acylcarnitines.

Fig. S2. The Ala levels over the four-year period (n = 90). DBSs with Ala levels of <100 nmol/mL (red circles) were excluded from the study because they were too low in comparison to the initial values at newborn period (12 samples).