



Comparison of Amino Acid Concentrations in Plasma and Dried Blood Spot Samples Using LC-MS/MS

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Abstract

Background: Dried blood spot samples are suitable for diagnosing some congenital errors of metabolism; however, they provide limited benefit in the regular monitoring of amino acids.

Objectives: The present study aimed to evaluate alanine (Ala), arginine (Arg), citrulline (Cit), glutamic acid (Glu), glycine (Gly), isoleucine (Ileu), leucine (Leu), methionine (Met), ornithine (Orn), phenylalanine (Phe), tyrosine (Tyr), and valine (Val) amino acid concentrations in dried blood and plasma samples obtained simultaneously.

Methods: Amino acid concentrations were determined in the plasma, and dried blood spot samples obtained simultaneously from 145 patients (50 females and 95 males). Amino acid concentrations in the plasma and dried blood spot samples were studied by LC-MS/MS using original kits.

Results: There were significant differences between dried blood spots and plasma in all amino acid concentrations, except for Met and Val. Bland-Altman analysis revealed the highest mean differences in Glu (-148.1), Gly (-70.1), and Ala (-58.1). Deming regression analysis showed that plasma and dried blood spot samples were consistent concerning Cit, Met, Phe, and Tyr concentrations.

Conclusions: Differences in methodology and sample can influence amino acid concentrations. Dried blood spot samples might cause errors in amino acid screening programs.

Keywords: Tandem Mass Spectrometry, Neonatal Screening, Inborn Errors of Metabolism, Amino Acids

1. Background

Amino acids, proteinogenic units, and derivatives have critical functions in biological processes, such as protein synthesis and metabolic pathways. Enzyme deficiencies in amino acid metabolism can alter their physiological concentrations resulting in clinical symptoms usually not specific to a single disease. Therefore, it is essential to identify free amino acid concentrations in physiological specimens because one or few compounds may act as biomarkers for a single or a group of metabolic disorders, eg, phenylalanine for phenylketonuria (PKU), ornithine, citrulline, and argininosuccinic acid for urea cycle disorders, and allo-isoleucine and valine for maple syrup urine disease (MSUD)(1). Free amino acid concentrations are used in clinical trials and for diagnostic purposes for evaluating patients' nutritional status (2).

Congenital metabolic disorders are a complex and heterogeneous group of genetic disorders that originate from a defect in the metabolic pathway and lead to malfunctioning metabolism and/or accumulation of toxic intermedi-

ate metabolites (3). Congenital metabolic disorders can occur at any age, manifest with non-specific clinical signs, and have a complex diagnostic process. They are associated with serious outcomes in clinical practice leading to morbidity and mortality, particularly in pediatrics. Although each disease is individually rarely encountered, the cumulative incidence is reported to be higher than one in 800 (4).

Measurement of amino acids in dried blood spot (DBS) has been widely used to identify newborns with various congenital disorders of amino acid metabolism, including PKU and MSUD (5). Collecting human blood through heel or finger puncture and dripping on a filter paper to obtain dried blood samples for analysis dates back to the early 1960s when Guthrie first used dried blood samples to determine phenylalanine concentration for the diagnosis of PKU in newborns (6). It is emphasized that dried blood samples have many specimen-related advantages over conventional blood, plasma, and serum samples (7-9). This novel way of blood collection has led to neonatal screen-

ing and other clinical tests (7, 8, 10). Metabolic screening of newborns with a Tandem mass spectrometer (MS/MS) using dried blood samples was first used by Millington et al. (11) for the analysis of acylcarnitines and Chace et al. (12) for the analysis of phenylalanine and tyrosine. Along with accelerated development in MS technology in the last decade, MS/MS systems with substantially improved precision and selectivity began to be used for quantitative analysis of various molecules in dried blood samples for the diagnosis of various hereditary metabolic disorders, as demonstrated through numerous practices in neonatal screening (13-17).

Dried blood spot samples are suitable for the diagnosis of some congenital errors of the metabolism; however, they provide limited benefits in regular monitoring of amino acids. Monitoring of plasma amino acids is frequently required to assess the efficacy of treatment in patients with PKU, MSUD, and some urea cycle defects (5).

2. Objectives

This study aimed to analyze the amino acid levels in simultaneously sampled dried blood spots and plasma by the liquid chromatography-tandem mass spectrometry (LC-MS/MS) method.

3. Methods

The study used the data of 145 patients (17 from the pediatrics ward, 70 from the endocrinology ward, 11 from the neonatal unit, and 47 from the pediatric intensive care unit) who applied to our hospital and had simultaneously sampled plasma and dried blood spots between January 2017 and December 2018. Of the patients, 50 were female, and 95 were male. This research project was approved by the Health Sciences University Diyarbakır Gazi Yaşargil Training and Research Hospital Ethics Committee (No. 27.09.2019 - 341).

The study samples were analyzed in an LC-MS/MS System (Zivak Technologies, Turkey) device using the original kits routinely used in our laboratory. For dried blood samples, amino acids were extracted from blood samples dried with an organic solvent containing internal standards. The extracted analytes were derivatized with reagents. Derivatized amino acids were analyzed in the LC-MS/MS system. The isotope dilution method was used to compute the results. Concentrations of the analytes were calculated according to the given internal standard areas.

For plasma amino acid concentrations, the samples were drawn into tubes containing dipotassium ethylenediaminetetraacetic acid (Becton Dickinson Vacutainer Systems, USA). Amino acid concentrations were studied in

plasma samples separated by appropriate centrifugation. Plasma amino acids were extracted from the samples by acidic extraction and deproteinization processes. The extracted analytes were derivatized with reagents. Derivatized amino acids were subjected to the volatilization process until they dried thoroughly. Then, they were dissolved again by adding reagents and analyzed in the LC-MS/MS system. The concentrations of standard solutions were plotted against the area of the standards. The signal value corresponding to each standard was included in the graphs to calculate the calibration curve. Unknown concentrations could be directly read from the function of the calibration curve.

The outcomes were alanine (Ala), arginine (Arg), citrulline (Cit), glutamic acid (Glu), glycine (Gly), isoleucine (Ileu), leucine (Leu), methionine (Met), ornithine (Orn), phenylalanine (Phe), tyrosine (Tyr), and valine (Val) amino acids, which are common in both methods. Since Leu and Ileu gave a total result in dried blood samples, a comparison was made by taking the sum of these two amino acids.

3.1. Statistical Analysis

Statistical analyses were done using SPSS and MedCalc package programs. The Kolmogorov-Smirnov test analyzed whether the data were distributed normally, and the Wilcoxon signed-rank sum test assessed the differences between the groups. A P-value < 0.05 was considered statistically significant. In addition, dried blood and plasma samples were compared using Bland Altman plots and Deming regression analysis.

4. Results

We used the data of 145 patients (50 females and 95 males) with amino acid concentrations simultaneously studied in dried blood and plasma samples. The mean age was 3.07 years (SD: 4.75, min: 0.01, max: 16.90) for females and 5.57 years (SD: 6.29, min: 0.01, max: 23.27) for males.

Table 1 illustrates the percentage coefficient of variation (CV) for all plasma and dried blood spot amino acids and their reference values. Amino acid concentrations of the patients are presented as mean \pm SD, median, minimum, and maximum in Table 2. While there was a significant difference between dried blood samples and plasma samples for Phe, Tyr, Ala, Arg, Cit, Glu, Gly, Leu+Ileu, and Orn amino acid concentrations, no significant difference was determined for Met and Val amino acids (Table 2).

In 74 patients whose reference values (3, 18) were consistent with their age group, plasma and dried blood amino acid concentrations were categorized as low, normal, and high, which were matched. Dried blood spot

Table 1. Coefficient of Variation (CV) and Reference Values of Plasma and Dried Blood Amino Acids

Features	ALA	ARG	CIT	GLU	GLY	LEU+ILEU	ILEU	LEU	MET	ORN	PHE	TYR	VAL
Plasma													
Inter-assay CV%	5	5.1	6.5	5.2	4.8	-	5.2	5	4.1	6.2	3.3	3.1	3.1
Intra-assay CV%	4.6	4.9	5.6	5.1	4.4	-	4.8	4.9	4.2	5.2	3.1	3.2	2.9
< 24 months ^a	139 - 474	29 - 134	9 - 38	31 - 202	111 - 426	-	31 - 105	48 - 175	11 - 35	20 - 130	28 - 80	26 - 115	83 - 300
2 - 17 years ^a	144 - 557	31 - 132	11 - 45	22 - 131	149 - 417	-	30 - 111	51 - 196	11 - 37	22 - 97	30 - 95	31 - 106	106 - 320
Dried blood spot													
Inter-assay CV%	3.3	3.6	4.9	4.8	3.8	3.9	-	-	3.5	2.2	3.7	3.5	3.2
Intra-assay CV%	3.3	3.1	4.5	5.4	4.1	3.4	-	-	3.3	2.3	4.2	3.1	3.5
< 4 days ^b	424-633	5.6 - 14.7	13.8 - 23	485 - 687.4	300 - 414	511 - 703	-	-	10.7 - 17.7	124 - 185	50.3 - 70.1	74.1 - 114.4	89.1 - 179.2
6 - 12 months ^b	253.5 - 355.8	14.2 - 23.5	18.5 - 25.6	194 - 306	146.3 - 251.6	305.4 - 440.1	-	-	9.4 - 21.9	69.3 - 122.5	40.5 - 62.7	52.6 - 85.5	118 - 160.3
1 - 3 years ^b	258.1 - 406.2	12.7 - 25.1	21.9 - 31.8	201.2 - 283.8	168.2 - 332.2	282.9 - 436.2	-	-	7 - 18.5	70.2 - 137.1	49.3 - 72.3	58.3 - 93.3	120.1 - 205.3
3 - 6 years ^b	293 - 386.9	13.8 - 25.8	22.1 - 31.8	214.8 - 262.3	157.1 - 229	275.3 - 396.1	-	-	8.2 - 15.6	82.4 - 111.3	47.1 - 84.2	50.1 - 91.3	103 - 174.4
6 - 12 years ^b	221.6 - 307.8	15.4 - 20.7	24.4 - 28.8	211 - 258.7	257.1 - 305.9	343 - 472.5	-	-	17.1 - 22.6	68.7 - 88.4	53.3 - 66.2	62.3 - 84.3	128.4 - 171.4

^a Reference values were obtained from Mayo Clinic Laboratories.^b Reference values were obtained from Uariyapanichkul et al. (3).**Table 2.** Amino Acid Levels of Patients

Amino Acids	Plasma Amino Acid Levels ($\mu\text{mol/L}$)				Dried Blood Amino Acid Levels ($\mu\text{mol/L}$)				P-Value ^a
	Mean \pm SD	Median	Min	Max	Mean \pm SD	Median	Min	Max	
Ala	384.75 \pm 244.06	337.97	102.17	1.793.86	442.82 \pm 215.45	418.24	1470	1.764.74	< 0.001
Arg	517 \pm 68.34	43.83	4.23	785.30	39.28 \pm 44.11	34.44	69	513.52	< 0.001
Cit	46.25 \pm 212.48	236	0.45	2.456.35	40.20 \pm 85.90	30.12	12.10	1500	< 0.001
Glu	78.19 \pm 496	67.72	6.81	270.87	226.34 \pm 126.32	200.43	86.60	8680	< 0.001
Gly	263.67 \pm 121.91	2430	865	1.185.26	333.77 \pm 174.33	2630	1420	935.75	< 0.001
Leu+Ileu	172.84 \pm 73.78	161.57	41.97	516.50	149.83 \pm 50.56	1420	62.70	3420	< 0.001
Met	42.58 \pm 97.85	29.19	9.65	1.165.16	39.67 \pm 76.56	29.10	13.80	924.45	0.751
Orn	1050 \pm 68.82	88.59	6.28	548.12	1271 \pm 84.56	95.40	40.60	5090	0.020
Phe	772 \pm 89.14	562	3.83	7477	87.58 \pm 97.17	670	32.25	963.37	< 0.001
Tyr	909 \pm 122.87	57.92	22.68	859.99	106.13 \pm 126.29	75.61	35.90	950.10	< 0.001
Val	190.41 \pm 72.85	189.18	35.69	512.45	190.41 \pm 59.31	181.11	66.90	4130	0.729

Abbreviation: SD, standard deviation.

^a Significant at P-value < 0.05. Wilcoxon signed-rank sum test.

Phe concentration was normal in four (36.4%) of 11 patients with high plasma Phe concentration, whereas dried blood Phe concentration was low in 13 (21.6%) and high in 14 (23.3%) of 60 patients with normal plasma Phe concentrations. Dried blood amino acid concentrations were normal in nine (36%) and low in six (24%) of 25 patients with high plasma Orn concentrations (Table 3).

The mean differences between plasma and dried blood spot concentrations in Bland-Altman analysis were as follows: Ala (-58.1), Arg (11.8), Cit (6.1), Glu (-148.1), Gly (-70.1), Leu+Ileu (23), Met (2.9), Orn (-22), Phe (-10.6), Tyr (-16.0), and Val (0.0). Accordingly, while plasma and dried blood spot samples showed similar results for Val, the plasma Ala, Glu, Gly, Orn, Phe, - Tyr concentrations were lower, and Arg, Cit,

Leu+Ileu, - Met concentrations were higher (Figure 1).

In Deming regression analysis, the confidence interval encompassed 1 for the slope and 0 for the intercept for Cit, Met, Phe, and Tyr. For other amino acids Ala, Arg, Glu, Gly Leu+Ileu, Met, Orn, and Val, the confidence interval did not include 1 and 0 values for the slope and intercept, respectively (Table 4). According to these data, Cit, Met, Phe, and Tyr amino acid concentrations appeared to be consistent in plasma and dried blood samples (Figure 2).

4. Discussion

Treatment of amino acid metabolism disorders allows avoidance of the toxic effects of dietary proteins and pro-

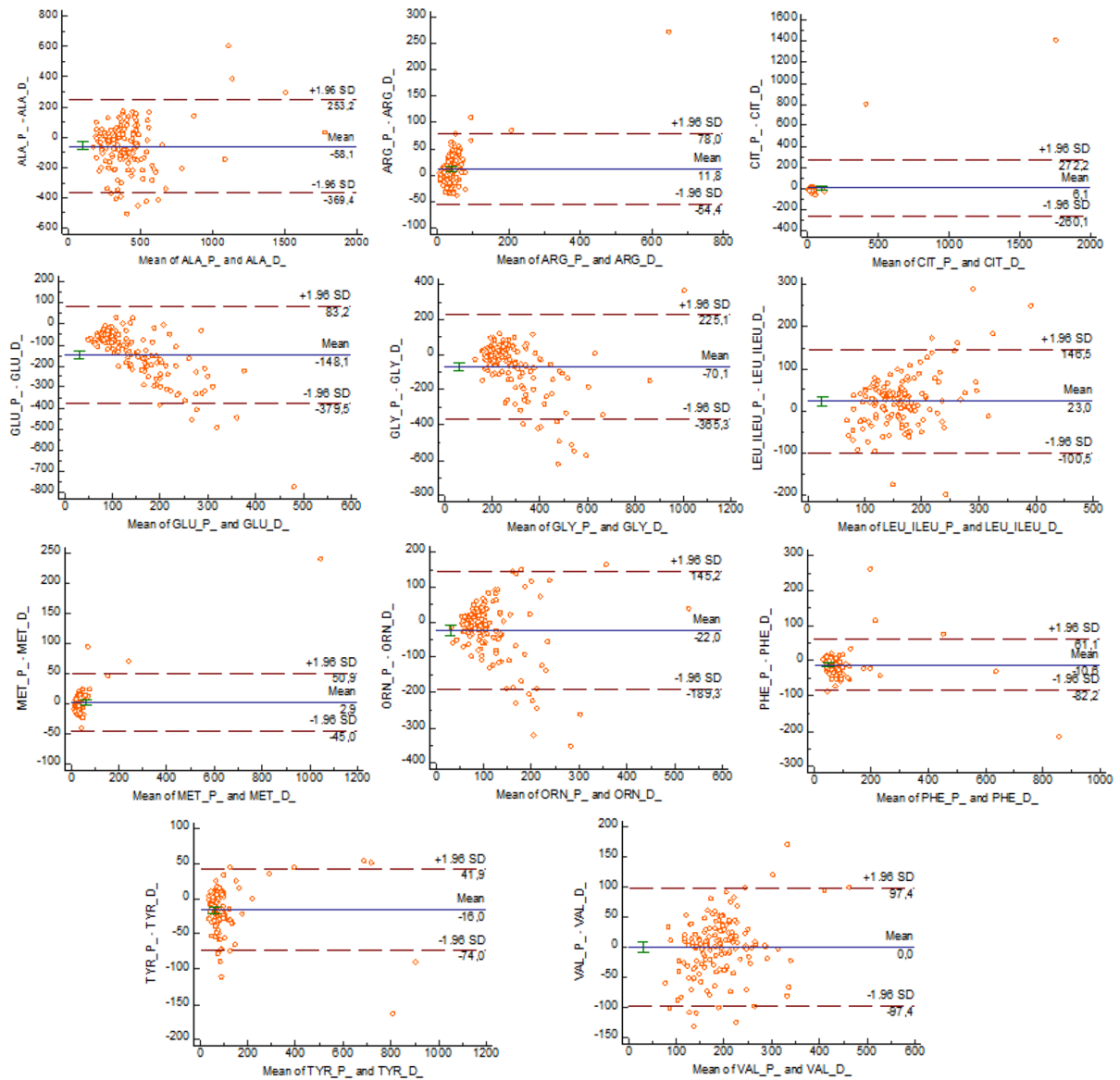


Figure 1. Bland-Altman plots of amino acids

vides adequate protein intake for normal growth and development. For this purpose, specific low-protein or low-amino acid diets are established. Patients with amino acid metabolism disorders require close monitoring of protein intake for appropriate growth that changes with age, development, and other factors (5). Volume/size and quality of DBS and hematocrit (Hct) can significantly affect the analytical results of amino acids and, therefore, may have important effects in monitoring hereditary metabolic disorders (19, 20). Within this context, detection and monitor-

ing of plasma amino acid concentrations are of critical importance.

Hct can substantially change according to age and gender, as well as the consequence of hydration status and because of diseases. Therefore, patient outcomes can show significant variations, and notably, DBS samples may not be the primary options for analysis in certain situations (21). Although neonatal screening programs are performed by MS/MS method in the USA, Canada, and many European countries, they are still not effectively per-

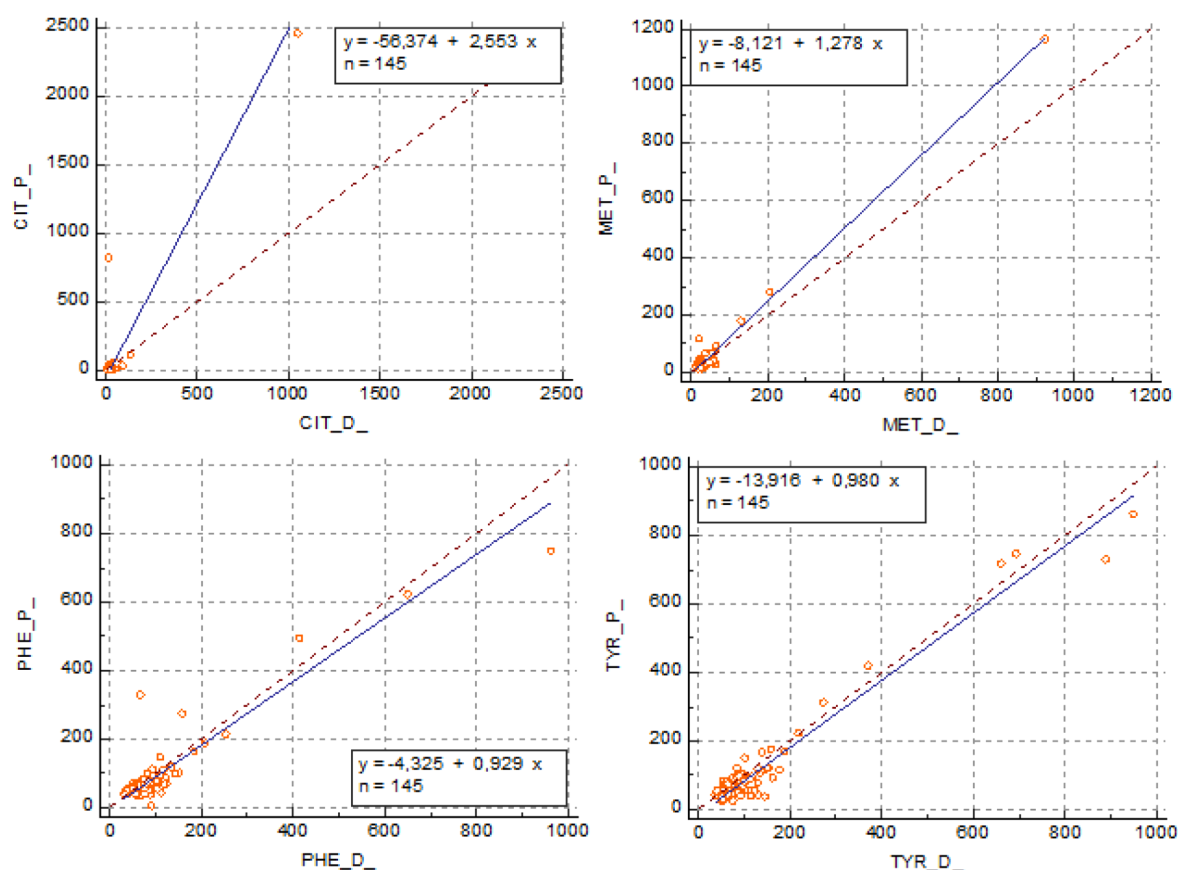


Figure 2. Graphs of amino acids with similar results in plasma and dried blood spot samples in Deming regression analysis

formed in some Middle Eastern, African, and Latin American countries (22, 23). Different neonatal screening programs are implemented worldwide. In Turkey, however, phenylketonuria is included in amino acid screening programs where the Phe level is studied by the fluorescent immunoassay method (24).

Gregory et al. (25) detected a significant difference between Phe concentrations in plasma analyzed by an HPLC amino acid analyzer and dried blood analyzed by MS/MS, reporting that amino acid concentrations were 15% lower in dried blood than in plasma. They suggested that one should be careful about the measurement method of plasma phenylalanine concentrations for dieting. On the contrary, Phe and Tyr concentrations in the present study were significantly higher in dried blood than in plasma. Although studies comparing amino acid concentrations in plasma and dried blood are limited, clinicians must keep the concentration differences in mind during patient follow-up. Kazanasmaz et al. found similar results for Phe, Tyr, and Phe/Tyr in plasma and dried blood samples of 224

patients. In this study, the specimens were analyzed by the LC-MS/MS method, reporting that dried blood samples give results with clinically adequate specificity and sensitivity (26). Unlike the study mentioned above, the present study found a significant difference between plasma and dried blood samples in Phe and Tyr amino acids. The mean Phe and Tyr concentrations were higher in dried blood samples.

Groselj et al. (27) compared amino acids in plasma measured by an HPLC amino acid analyzer and dried blood measured by MS/MS, finding that dried blood showed lower concentrations by 26.1% for Phe and 15.5% for Try. They stated that the differences could be due to the methods used to diagnose and clinical monitoring of hyperphenylalaninemia patients. Significant differences between plasma and dried blood Phe and Tyr might have resulted from the differences in specimens or methods, which should be considered in the patient diagnosis and follow-up.

In Bland Altman's analysis, the mean difference was the

Table 3. Comparison of Plasma and Dried Blood Amino Acids in Patients with Age-matched Reference Values (N = 74)

Amino Acids/ Plasma Levels	Dried Blood Levels			Total
	Low	Normal	High	
ALA				
Low	0	7	2	9
Normal	25	7	27	59
High	0	1	5	6
ARG				
Low	1	8	12	21
Normal	1	11	39	51
High	0	0	2	2
CIT				
Low	3	1	3	7
Normal	8	11	41	60
High	0	0	7	7
GLU				
Low	4	0	1	5
Normal	33	17	16	66
High	2	1	0	3
GLY				
Low	1	1	3	5
Normal	16	26	25	67
High	0	0	2	2
LEU+ILEU				
Low	4	0	0	4
Normal	63	0	0	63
High	6	1	0	7
MET				
Low	0	0	1	1
Normal	47	7	0	54
High	0	0	19	19
ORN				
Low	0	0	1	1
Normal	21	19	8	48
High	6	9	10	25
PHE				
Low	0	1	2	3
Normal	13	33	14	60
High	0	4	7	11
TYR				
Low	2	0	0	2
Normal	21	29	16	66
High	0	1	5	6
VAL				
Low	0	2	0	2
Normal	1	30	38	69
High	0	0	3	3

highest at 148.1 for Glu, 70.1 for Gly, and 58.1 for Ala. Plasma and dried blood amino acid concentrations were evaluated in 74 patients with matched reference values. Dried blood amino acid concentrations were normal in four (36.4%) of 11 patients with high plasma Phe concentrations. The high mean differences between plasma and dried blood samples and inconsistencies between reference in-

tervals for amino acids indicate that there may be errors during clinical decision-making. Although Wilcoxon signed-rank sum test revealed no significant difference between Val and Met amino acids, the samples were not compatible in terms of method consistency according to Deming regression. Nevertheless, Deming regression revealed that plasma and dried blood samples were compatible for Cit, Met, Phe, and Tyr amino acids.

The present study showed that amino acid concentrations showed significant differences between plasma and dried blood samples. Therefore, as comparative studies about amino acids are lacking, there may be specimen-related or methodological differences for patients, which should be considered in clinics. We think that it would be appropriate to follow plasma amino acids in order not to ignore the differences between the samples. The lack of clinical follow-ups in this study could be a significant limitation. In addition, further studies may consider the potential biochemical and clinical variables affecting plasma and dried blood spot amino acids in patients. Thus, more precise information can be obtained about the source of differences between the samples and their clinical usefulness.

Better outcomes are achieved in the identification of congenital metabolic disorders and amino acid concentrations with improving technology. Both methodological and specimen-related features influence amino acid concentrations, and DBS samples may cause errors even in screening programs. For this reason, clinicians and laboratories should consider that deviations in the specimens and systems might be crucial for the diagnosis and monitoring of metabolic diseases.

Footnotes

Authors' Contribution: Study concept and design: Ö. A.; Analysis and interpretation of data: Ö. A.; Drafting of the manuscript: Ö. A.; Critical revision of the manuscript for important intellectual content: Ö. A. and R.E.C.E; Statistical analysis: Ö. A.

Conflict of Interests: The authors declare that they have no competing interests.

Data Reproducibility: The data presented in this study are openly available in one of the repositories or will be available on request from the corresponding author by this journal representative at any time during submission or after publication. Otherwise, all consequences of possible withdrawal or future retraction will be with the corresponding author.

Ethical Approval: This research project was approved by the Health Sciences University Diyarbakır Gazi Yaşargil

Table 4. Deming Regression Results

Amino acids	Equation	Intercept (95% CI)	Slope (95% CI)	r
Ala	$y = -94.8628 + 1831 x$	-94.8628 (-189.9381 to 0.2125)	1831 (0.8463 to 1.3199)	0.77
Arg	$y = -8.7445 + 1.5229 x$	-8.7445 (-22.6027 to 5.1136)	1.5229 (1.1541 to 1.8917)	0.91
Cit	$y = -56.3737 + 2.5530 x$	-56.3737 (-10015.1099 to 9902.3624)	2.5530 (-297.6161 to 302.7221)	0.93
Glu	$y = -15.7609 + 0.4151 x$	-15.7609 (-83.6542 to 52.1325)	0.4151 (0.8597 to 0.7442)	0.36
Gly	$y = 87.9261 + 0.5265 x$	87.9261 (-27.2085 to 203608)	0.5265 (0.1419 to 0.9112)	0.53
Met	$y = -8.1214 + 1.2785 x$	-8.1214 (-18434 to 1.8005)	1.2785 (0.9857 to 1.5713)	0.99
Leu+Ileu	$y = -43.3491 + 1.4429 x$	-43.3491 (-126.6931 to 39.9949)	1.4429 (0.8466 to 2.393)	0.54
Orn	$y = 59.5131 + 0.3581 x$	59.5131 (19.9630 to 99633)	0.3581 (-0.02288 to 0.7186)	0.40
Phe	$y = -4.3254 + 0.9288 x$	-4.3254 (-35.4172 to 26.7664)	0.9288 (0.5177 to 1.3399)	0.93
Tyr	$y = -13.9163 + 0.9800 x$	-13.9163 (-26.9470 to -0.8855)	0.9800 (0.8326 to 1.1274)	0.97
Val	$y = -63.7035 + 1.3346 x$	-63.7035 (-111.6221 to -15.7850)	1.3346 (1.756 to 1.5935)	0.74

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