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Age-Specific Reference Values for Amino Acid Content in Dried Blood Spots in Children in Western Kazakhstan, Measured by Tandem Mass Spectrometry

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Abstract: Measuring the level of amino acids in the blood is one of the stages in the early diagnosis of inborn errors of metabolism (IEM), implying timely initiation of therapeutic measures. Tandem mass spectrometry (MS/MS) is now replacing traditional IEM screening methods. Dried blood spot amino acid reference values developed for the pediatric population are crucial for interpreting test results and diagnosing aminoacidopathies. The study aims to establish reference values for amino acid (AAs) concentrations in samples of dried blood spots from newborns in Western Kazakhstan using LC-MS/MS (liquid chromatography-tandem mass spectrometry) technology. Methods: The cross-sectional study included 250 healthy newborns of Western Kazakhstan aged 1-3 days, born at term and breastfed, 49.2% male and 50.2% female. To establish the age-specific reference values for AAs, newborns were divided into three groups: (1) 1 day, (2) 2 days, and (3) 3 days. Blood samples on Guthrie cards were collected on days 1–3 of life and quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Nonparametric statistical approaches were used to generate the 2.5th–97.5th percentile distributions for newborns. Results: 3 of the 15 DBS amino acid distributions were dependent on gender. There was a statistically significant difference in the mean level of alanine, citrulline, and glutamic acid in males and females. The highest values were determined in the female group. Age-related differences in glutamic acid, leucine, ornithine, tyrosine, and valine concentration levels were observed. No significant correlations were found between the concentrations of 15 amino acids in dried blood spots and the body weight of newborns. Conclusion: The present study established amino acid concentrations that can be utilized as reference standards in Kazakhstan's newborn screening program for inherited metabolic diseases.

Keywords: Newborn screening, Amino acids, Dried blood spots, Tandem mass spectrometry Reference values.

Introduction

Measuring amino acid (AAs) levels in the blood is essential for the early diagnosis of inborn errors of metabolism (IEM), implying the timely commencement of therapeutic measures. The low incidence and prevalence of IEM cause them to be poorly studied and challenging to diagnose and treat (Céspedes et al., 2017). A significant problem with IEM is delayed diagnosis (5-10 years) or misdiagnosis due to the lack of specialized laboratories that perform accurate tests, resulting in delayed or lack of treatment. Early diagnosis of IEM can significantly reduce the risk of death and prevent long-term neurological complications (Scolamiero et al., 2015; Uaariyapanichkul et al., 2018).

Currently, tandem mass spectrometry (MS/MS) replaces traditional screening methods, which usually analyze individual biomarkers for each disease. This method can detect and quantify multiple IEM in a single blood spot

for most fatty acid oxidation disorders, aminoacidopathies, organic acid disorders, and urea cycle disorders (Mak et al., 2013; Uaariyapanichkul et al., 2018; Gelb et al., 2022).Dried blood spots (DBS) are usually the deposition of small volumes of capillary blood on special paper cards. Compared to whole blood or plasma samples, their advantages are that sample collection is more accessible, and there are no problems storing and transporting samples (Wagner et al.,2016). Reference values are crucial for interpreting test results and making a diagnosis (Lepage et al., 2006; Teodoro-Morrison et al., 2015; Adeli et al., 2017). Using carefully designed amino acid reference intervals appropriate for age, gender, and geographic location can facilitate the diagnosis of a wide range of disorders of physical, sexual, metabolic, and neurological development (Dietzen et al., 2016).

Pediatric populations require standards that reflect rapid physiological changes associated with growth, but these are often difficult to establish due to challenges related to obtaining sufficient samples from healthy children (Dietzen et al., 2016). Due to limited access to healthy controls in the pediatric population, laboratory data can provide information for estimating reference ranges (Dogan et al., 2017). New studies related to reference values of AAs in the blood of children are emerging all over the world, but most of them describe reference intervals for blood plasma (Yi et al., 2011; Méndez et al., 2013; Macit et al., 2014; Haschke-Becher et al., 2016).

In Kazakhstan, screening newborns using the MS/MS method is not mandatory, and there are no developed reference intervals for the concentration of AAs in DBS for different age groups of the pediatric population, including newborns. We initiated selective screening to obtain data on the frequency of IEM in children at risk in Western Kazakhstan. The results of selective screening tests in different age groups of the examined children should be interpreted by comparison with the reference values and/or threshold levels established for these groups. Therefore, one of the tasks is to establish reference intervals for the concentration of AAs in DBS in newborn children of Western Kazakhstan.

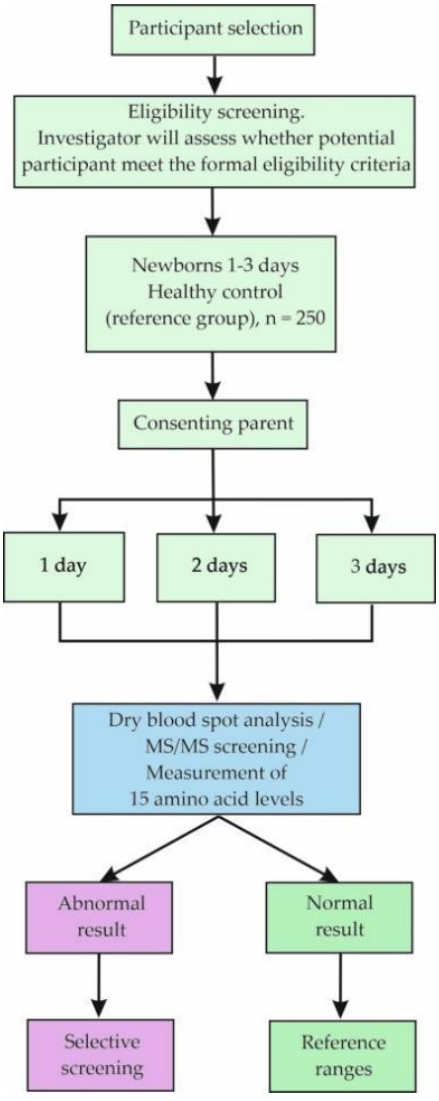

Figure 1. Study flowchart

Study Objectives

To establish reference values for AA concentrations in samples of DBS from newborns in Western Kazakhstan using LC-MS/MS (liquid chromatography-tandem mass spectrometry) technology.

Tasks

1. To set reference ranges of AA concentrations in samples of DBS of 250 newborns of Western Kazakhstan aged 1-3 days using LC-MS/MS technology.
2. To evaluate factors that may affect AA levels.
3. To compare findings of the determined analytes in newborns of Western Kazakhstan DBS with the results of previously published studies in other populations.

Methods

Data Sources

The data of this study were obtained during the examination of 250 healthy newborns aged 1-3 days to establish reference values of 15 AAs (Figure 1). The study was approved by the Bioethics Committee of the West Kazakhstan Marat Ospanov Medical University (Ref. No. 7, 09/09/2020.) Written informed consent (IS) was obtained from the parents and/or legal guardians of children after birth to collect a DBS sample. Demographic and anthropometric data of newborns are presented in Table 1.

Table 1. Demographic and anthropometric data of study participants

	Healthy newborns (n=250)			
	Group A 1 day (n=36)	Group B 2 days (n=116)	Group C 3 days (n=98)	The whole sample
Weight in grams, Median (IQR)	3434 (3180;3560)	3600 (3300;3833)	3615 (3470;3860)	3560 (3298;3830)
Gender				
Male, n, %	16 (44,4 %)	63 (54,3 %)	44 (44,9 %)	123 (49,2 %)
Female, n, %	20 (55,6 %)	53 (45,7 %)	54 (55,1 %)	127 (50,2 %)
Geographic distribution				
Urban population, n, %	22 (61,1 %)	68 (58,6 %)	54 (55,1 %)	144 (57,6 %)
Rural population, n, %	14 (38,9 %)	48 (41,4 %)	44 (44,9 %)	106 (42,4 %)

Criteria for Inclusion in the Study

Pediatricians examine all children in this study to ensure they do not suffer from any disorder or chronic disease. Healthy male and female newborns born after an uncomplicated pregnancy and vaginal delivery should have a body weight of 2500–4000 g, gestational age of 37–42 weeks, and an APGAR score greater than 7 in 10 minutes after birth. None should be diagnosed with birth asphyxia, defined as an Apgar score ≤6 at 5 min. All newborns must be breastfed, and their mothers must be healthy between 24 and 36. They must not have any food restrictions (vegetarian, vegan, etc.). Echograms of the placenta and fetus, as well as laboratory tests, should be normal throughout pregnancy.

Mass Spectrometry Analysis

Specimen Collection and Storage

Neonatal whole blood samples were collected from infants no earlier than 3 hours after feeding by heel prick using a heel stick. Five drops of whole blood (each ~75 µl) were applied to Guthrie cards, Ahlstrom 226 filter

paper, and PerkinElmer 226 Five-Spot Card (PerkinElmer Health Sciences, Greenville, USA) to form dried blood spots (DBSs) for LC-MS/MS analysis. Samples were dried for 4 hours at room temperature and then stored at 4°C in labeled individual zip-lock plastic envelopes with desiccants until analyzed by LC-MS/MS. Samples were sent to the laboratory within five days. In the case of long-term storage of samples, it was carried out at a temperature of –20°C.

Specimen Preparation and LC-MS/MS Analysis

The Neobase2 TM Non-derivatized MSMS kit (PerkinElmer, Wallac Oy, Turku, Finland) was used to quantify 15 amino acids in DBS according to the manufacturer's instructions. Vial with lyophilized isotope-labeled internal standards (IS) containing 2H3-Alanine (Ala IS), 2H4, 13C-Arginine (Arg IS), 2H2-Citrulline (Cit IS), 13C5-Glutamine (Gln IS), 13C5-Glutamic acid (Glu IS), 15N,2-13C-Glycine (Gly IS), 2H3-Leucine (Leu IS), 2H3-Isoleucine (Leu IS), 2H3-Hydroxyproline (Leu IS), 2H3-Methionine (Met IS), 2H6-Ornithine (Orn IS), 13C6-Phenylalanine (Phe IS), 13C5-Proline (Pro IS), 13C6-Tyrosine (Tyr IS), and 15N-13C5-Valine (Val IS) was being recovered by adding 1.4 ml of the extraction solution that is included in the Neobase 2 kit. The Extraction Working Solution (EWS) IS was prepared by diluting the recovered internal standards with the extraction solution of 1:100 (v/v).

DBS were analyzed using a Shimadzu LCMS-8050 Triple Quadrupole Mass Spectrometer (Shimadzu Corporation, Kyoto, Japan). Sample preparation was based on extraction followed by derivatization into oil esters. Level I and Level II (low standard and high standard) dried blood drops were included with each assay lot of the Neobase2 TM Non-derivatized MSMS kit to monitor system accuracy and precision.

To analyze amino acids and acylcarnitines, stored DBS card samples are brought to room temperature (+18 to +25°C) before extraction. A 3.2 mm disc (equivalent to ~3.1 µl of whole blood) is punched out of one dried blood spot with a diameter of 3.2 mm using a Wallac DBS Puncher (PerkinElmer, Wallac Oy, Mustionkatu 6, FI-20750 Turku, Finland) into the well of the 96-well polystyrene U-bottom microplate supplied with the Neobase2 TM Non-derivatized MSMS kit. After adding 125 µL of working extraction solution to each well of the microplate, the plate is covered with an adhesive aluminum film and incubated for 30 minutes at room temperature on a microplate shaker with a shaking speed of 650 rpm.

After incubation, 100 µL of the supernatant is transferred to a new 96-well U-bottom microplate, covered with aluminum foil to reduce evaporation, and incubated for 1 hour. The plate is then placed into the Shimadzu LCMS-8050 Triple Quadrupole Mass Spectrometer autosampler, and 5 µL of supernatant is injected into the LCMS for analysis.

Statistical Analysis

Shapiro-Wilk and Kolmogorov-Smirnov tests were used to check the normality of the distribution. The data obtained in the study demonstrated that the distribution of amino acids in DBS differs from normal. Me (median) and quartiles (IQR interquartile range) were used for descriptive statistics of the samples. Nonparametric tests (Mann-Whitney U test, Kruskal-Wallis H test) were used to test differences in AA concentrations depending on various factors (gender, age, place of residence).

Reference intervals in the group of healthy newborns aged 1-3 days were determined non-parametrically and corresponded to the 2.5-97.5th percentile of the experimental distribution. Considering the skewed distribution, correlations between body weight, age, and the concentration of amino acids in dry blood spots were performed using Spearman's test. Two-sided levels <0.05 are assumed to be statistically significant. Statistical analysis was done using the software IBM SPSS v. 23.0 (IBM, Armonk, NY, USA) and Statistica (StatSoft, Inc., Tulsa, OK, USA, v. 10).

Results and Discussion

Descriptive statistics and reference intervals for the concentration of 15AAs in whole blood of healthy newborns divided into subgroups according to age are presented in Table 2. For each analyte, the upper cut-off limit is set above the 97.5th percentile, while the lower limit is set below the 2.5th percentile.

Table 2. Amino acid levels in dried blood spots of 250 healthy newborns aged 1-3 days in Western Kazakhstan.

Amino acid, µmol/l		All children 1-3 days (n = 250)	Group A 1 day (n = 36)	Group B 2 days (n = 116)	Group C 3 days (n = 98)	Kruskal–Wallis *H* test	p-values
5-Oxo Pro	Median	86.68	99.23	86.68	83.83	1.26	0.532
	Range	53.89;108.87	69.90;108.87	46.51;111.34	58.57;107.40		
	2.5th-97.5th	22.70–159.87	22.64–68.93	22.24–165.99	22.70–156.05		
Ala	Median	265.01	270.11	254.66	285.48	2.46	0.292
	Range	213.91;315.71	199.07;301.89	211.54;306.94	223.70;325.32		
	2.5th-97.5th	146.27–422.57	137.55–371.65	161.94–461.45	146.27–422.58		
Arg	Median	18.13	18.46	17.02	19.17	8.96	0.013
	Range	15.56;22.68	15.85;22.73	14.12;21.65	16.07;22.68		
	2.5th-97.5th	11.63–33.14	12.45–33.01	10.42–32.94	13.11–35.33		
Cit	Median	19.56	18.51	19.13	19.58	0.591	0.744
	Range	15.97;22.65	16.54;22.39	15.55;23.32	16.83;22.41		
	2.5th-97.5th	11.76–30.54	11.64–26.25	11.76–30.54	12.41–31.15		
Gln	Median	411.97	415.11	417.74	402.29	1.95	0.376
	Range	352.17;441.54	374.88;490.63	385.32;501.08	317.02;496.21		
	2.5th-97.5th	201.24-648.33	208.75-678.42	215.30-685.40	189.13-674.83		
Glu	Median	428.80	477.05	390.92	438.17	15.74	0.0004
	Range	337.16;490.41	447.17;512.47	305.30;473.20	326.59;505.28		
	2.5th-97.5th	207.49–653.27	271.16–653.27	174.23–626.72	229.03–660.15		
Gly	Median	536.06	514.99	522.46	537.90	1.87	0.393
	Range	473.46;631.52	408.39;632.03	464.63;631.52	485.19;629.45		
	2.5th-97.5th	343.23–873.33	351.02–846.68	307.20–936.85	375.04–749.72		
Ile	Median	58.05	55.26	54.13	59.89	3.44	0.199
	Range	53.21;64.62	50.87;62.67	45.35;60.41	54.29;72.30		
	2.5th-97.5th	42.76 – 89.17	36.72 – 85.14	34.29 – 83.45	43.82 – 98.41		
Leu	Median	149.58	163.95	142.72	165.58	18.34	0.0001
	Range	129.44;178.26	135.48;171.08	125.03;162.33	136.12;196.07		
	2.5th-97.5th	96.04–231.24	114.04–219.71	88.24–225.64	115.90–257.35		
Met	Median	25.09	27.38	24.31	24.50	5.80	0.055
	Range	21.62;27.93	24.32;30.03	21.44;27.86	21.62;27.09		
	2.5th-97.5th	13.39–37.84	13.39–37,84	15.55–40.71	13.14–37.56		
Orn	Median	106.95	95.18	92.67	135.94	29.69	0.0000
	Range	80.98;144.38	76.90;130.03	73.00;117.92	105.48;162.91		
	2.5th-97.5th	49.31–205.45	49.31–166.99	45.81–226.01	66.42–205.45		
Phe	Median	60.44	62.82	60.62	58.82	3.11	0.212
	Range	52.98;67.63	50.99;70.78	54.21;70.17	50.90;66.71		
	2.5th-97.5th	43.22–92.43	43.83–92.43	40.79–108.62	43.36–85.37		
Pro	Median	159.15	145.79	160.72	157.35	1.18	0.553
	Range	139.75;185.77	136.44;191.58	140.50;182.36	139.75;192.37		
	2.5th-97.5th	106.43–246.79	115.25–228.15	103.25–260.13	106.43–246.79		
Tyr	Median	106.08	140.71	95.03	111.15	15.68	0.0004
	Range	81.93;141.62	97.90;164.04	78.62;122.72	84.83;139.68		

	2.5th-97.5th	52.53–248.17	58.72–298.39	37.78–252.27	54.99–205.94		
Val	Median	127.74	133.86	121.66	138.99	14.96	0.0006
	Range	107.47;151.18	101.68;154.18	100.95;141.93	115.70;161.42		
	2.5th-97.5th	85.41–194.93	80.51–187.07	71.50–195.81	98.22–194.93		

Differences in the distribution of amino acid levels in DBS between groups of newborns aged 1, 2, and 3 days, determined using the Kruskal-Wallis test, are recorded in Table 2. Statistically significant differences between age groups are noticed in the concentration of arginine, glutamic acid, leucine, ornithine, tyrosine, and valine (Table 2). In addition, significant weak positive correlations with age were established for the concentrations in DBS of leucine, ornithine, and valine, and weak negative correlations for methionine (Table 3).

Table 3. Statistical analysis according to age (Spearman's correlation) and gender (Mann-Whitney U test).

Analyte	Spearman correlation		Male N=123		Female N=127		p-values
	ρ	p-values	Median (μmol/L)	Range	Median (μmol/L)	Range	
5-Oxo Pro	-0.054	0.397	88.53	58.57;117.73	85.13	51.36;106.23	0.197
Ala	0.099	0.132	259.74	206.52;301.03	271.30	221.76;325.83	0.031
Arg	0.083	0.210	18.27	15.04;22.73	18.04	15.71;22.45	0.934
Cit	0.043	0.502	18.52	15.75;21.48	20.54	16.23;24.26	0,011
Gln	-0.023	0.668	413.74	362.55;459.57	408.10	321.17;435.49	0.502
Glu	-0.078	0.220	422.20	326.59;472.78	444.37	342.12;505.39	0.018
Gly	0.083	0.192	517.92	456.78;629.45	536.28	483.27;632.03	0.177
Ile	-0.041	0.305	61.44	58.08;68.07	56.30	47.58;60.31	0.121
Leu	0.173	0.007	155.48	129.46;184.30	146.71	127.61;171.08	0.128
Met	-0.126	0.047	24.62	21.04;27.57	25.69	22.22;29.13	0.071
Orn	0.304	0.000	105.69	81.83;149.17	108.79	79.09;143.02	0.845
Phe	-0.114	0.071	59.47	51.10;69.22	60.54	54.24;67.54	0.623
Pro	0.057	0.366	154.43	132.85;184.31	160.51	143.76;192.37	0.085
Tyr	-0.063	0.319	104.23	83.81;131.38	111.15	80.17;144.50	0.673
Val	0.189	0.003	128.92	104.06;148.56	125.82	109.37;154.93	0.849

Significant differences between female and male newborns were established by the concentration of alanine, citrulline, and glutamic acid in DBS (Table 3). In a study by Manta-Vogli et al. (2020), which assessed the concentration of amino acids involved in neurotransmission, statistically significant differences were also found between male and female newborns in the level of glutamic acid in DBS.

When assessing the effect of newborn body weight on the level of amino acids, no significant correlations were found between the concentration of 15 amino acids in dry blood spots and newborn body weight.

The levels of amino acids, as well as other metabolites in the blood, are influenced by several continuous and categorical variables, such as gestational age, birth weight, gender, ethnicity, age at blood collection, nutritional therapy (feeding pattern) and birth season, which have been shown to influence screening accuracy (Blanco et al., 2011; Ryckman et al., 2013; Hall et al., 2014; Clark et al., 2014; Peng et al., 2020). Environmental and nutrient changes over time, particularly protein intake, may affect amino acid levels in young children (Haschke-Becher et al., 2016).

We compared the results of measuring amino acid levels in DBS in newborns of western Kazakhstan with the findings of previously published studies in other populations (Dietzen et al., 2016; Haynes et al., 2016; Céspedes et al., 2017; Uaariyapanichkul et al. 2018; Yu et al., 2018; Sarker et al., 2019; Tan et al., 2021). Many researchers confirmed the relationship between gender and the level of certain AAs in DBS (Dietzen et al., 2016; Manta-Vogli et al., 2020; Uaariyapanichkul et al., 2018). The relationship between amino acid levels and birth weight was confirmed by Yu et al. (2018) and Manta-Vogli et al. (2020) but denied by Dietzen et al. (2016).

Conclusion

The present study established amino acid concentrations that can be applied as reference standards in Kazakhstan's newborn screening program for inherited metabolic diseases.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS Journal belongs to the authors.

Acknowledgements or Notes

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