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Determining the Reference Range of Amino Acids in Healthy Neonatal Blood Samples in Northeast Iran Using LC-MS/MS

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Abstract

Background: Amino acid analysis is an important tool for the diagnosis of metabolic disorders in newborns. Today, Liquid Chromatography tandem mass spectrometry (LC-MS/MS) has emerged as a powerful technique for amino acid analysis. We aimed to determine the local normal range of amino acids in dried blood spot (DBS) samples of neonates using LC-MS/MS.

Methods: A total of 1005 samples from healthy neonates of northeast and east of Iran aged 2-7 days were utilized for normal range determination. The amino acids were extracted from dried blood spot samples using organic solvent and then analyzed using LC-MS/MS system. The 1%, 2.5%, 97.5%, and 99% percentiles were calculated, and the results were compared to the global cut-off values.

Results: The results showed that glutamic acid has the highest concentration range among amino acids evaluated in this study (178.94 – 421.31μmol/L). Moreover, the plasma concentrations of Glycine (142.65 – 397.06 μmol/L), Alanine (97.00–349.72 μmol/L), Proline (63.77 – 236.53 μmol/L), and Tyrosine (25.79 – 150.58 μmol/L) were in the next ranks. Comparing the obtained results with the global values obtained in the R4S study indicated a slight difference between the obtained local normal values and the global values.

Conclusion: The calculated values were slightly different from global values obtained in the R4S study and regional values calculated in other studies. This further emphasized the importance of the local establishment of reference values, which facilitates the correct interpretation and diagnosis in the Newborn Screening Programs.

Keywords: Amino acids, Dried blood spots, Inborn Errors of metabolism, LC-MS/MS, Newborn screening.

Introduction

Amino acids are small biological molecules that together constitute different nitrogen-based compounds like proteins. Amino acids have various biochemical structures that provide notable structural and functional diversity in proteins (1, 2). ased on the ability of the body to synthesize amino acids, they are usually categorized into two groups: non-essential amino acids, which are typically synthesized by the body, and essential amino acids which are not synthesized by the body. These essential amino acids must be supplied by the diet and are vital for the maintenance of health and growth (3).

Inborn errors of metabolism (IEMs) are a group of complex genetic metabolic disorders with high heterogeneity either in phenotype or in the genotype (4). Generally, these disorders are caused by mutations in the genes encoding enzymes contributing to metabolic pathways.

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Due to the heterogeneous character of these disorders, and as a result of the deficient or mutated activity of the intermediary proteins and enzymes of metabolic pathways, toxic intermediate compounds accumulate and usually cause a wide spectrum of diseases with various clinical presentations in patients (4-6). The most common clinical manifestations of IEMs are growth failure and retardation, failure to thrive, weight loss, encephalopathy, stroke, precocious or delayed puberty, developmental delay, seizures, abnormalities of the immune system, dementia, skin rash, vomiting, diarrhea, abdominal pain, heart failure, edema, dehydration, hypertension, liver failure (7, 8).

In recent decades, with the development of new diagnostic methods and technologies, numerous novel IEMs have been identified (9). IEMs are rare disorders individually but due to their versatility, the collective global prevalence rate of IEMs is high and varies between 1 in 800-2500 live births (4, 10, 11). It is noteworthy that IEMs usually present with non-specific clinical manifestations, and they usually appear in the neonatal period or infancy; however, they are not age-dependent and may also manifest at any age of life (11, 12). About 30% of IEMs present with the involvement of the nervous system, and considering the severity of clinical consequences, morbidity, and mortality are seen, especially in the pediatrics (13).

Regarding the diagnosis of IEMs, early diagnosis can directly affect the quality of life in IEM patients since early diet management along with immediate treatment, remarkably decreases the severity and symptoms of IEMs provides physicians with the data to taper the diagnosis pathway and facilitate the differential diagnosis (9). Today, routine Newborn Screening tests (NBS) are able to detect the majority of genetically recessive metabolic disorders in the blood, plasma, and urine samples, especially using mass chromatographic methods (14, 15). This leads to earlier treatment and diet supplementation and, finally, better clinical outcomes in patients.

Currently, high-performance liquid chromatography (HPLC) and LC-MS/MS methods are widely used for IEM diagnosis around the world (16). Although HPLC is a reliable method, there are some disadvantages, including long analysis time, limited evaluated amino acids, and high variation between different laboratories (17). In contrast to HPLC, LC-MS/MS samples can be applied for assessing multiple analytes with a small amount of sample (blood) in less than two minutes. Additionally, the variation in results and false positives are less in LC-MS/MS methods compared to HPLC. Therefore, nowadays LC-MS/MS technique is considered more reliable for IEM diagnosis (12, 14).

In certain regions of Iran with high consanguinity, the incidence of inherited and congenital disorders indicates an increasing pattern (18). Hence, early diagnosis is pivotal for starting treatment in order to reduce the severity of symptoms. Several factors influence the level of amino acids in the blood, including race, age, sex, illness, nutritional status, and fasting time (11, 19, 20). Valid reference values are fundamental for accurate clinical interpretation and diagnosis, particularly in pediatrics, since reference values in this population should reflect the situation related to growth and development (21). Therefore, in the present study, we aim to establish reliable LC-MS/MS-based reference intervals for amino acids in neonates in Mashhad, Razavi Khorasan Province, Iran.

Materials and Methods

A total of 1005 samples were taken from healthy neonates aged 2-7 days old and referred to Pardis Clinical and Genetic Laboratory (Regional Diagnostic Laboratory of the Northeast of Iran), Mashhad, Iran. Pardis Laboratory is the referral laboratory for the diagnosis of congenital metabolic disorders in Razavi Khorasan Province which is located in Mashhad. The sample selection was performed based on the following criteria: The neonate must be breastfed by his/her mother, must not use drugs like Alborak acid, or must not been

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diagnosed with a disease. Due to some interactions, a full-fat diet is also prohibited.
The whole blood sample was collected from children's feet using the heel prick method and rapidly spotted onto Whatman filter cards. Then DBSs were analyzed in terms of amino acid levels using the LC-MS/MS system. Patient data were analyzed with SPSS software (version 16).

Next, to calculate the reference intervals, we employed two methods. The first method uses 2.5% and 97.5% percentiles as lower and higher values and considers the central 95% of the normal data for reference intervals. The second calculation method used non-parametric tests. In order to determine the most appropriate method for reference interval calculation, we employed the bootstrapping method (22).

All the experiments and procedures were performed in accordance with the ethical standards of the Committee on Human Experimentation of Mashhad University of Medical Sciences (MUMS). When patients were referred to the laboratory, the informed parental consent was filled out by the neonates' parents and collected from them.

Results

Descriptive statistics

To establish a reliable reference range for amino acids in neonates, the amino acid profiles of 1005 healthy neonates were obtained, and the amount of each amino acid was measured in DBS samples by an LC-MS/MS system. The calculations were done according to the globally established methods in laboratories, and several variables, including median, mean, standard deviation (SD), confidence intervals (2.5% and 97.5%), and coefficient of variation (CV) were calculated from these data.
The highest and lowest values were calculated using the following formulas:
High value = Median + X SD
Low value = Median – X SD
Factor X was calculated using the data provided by the R4S study (23). The summary of the analysis and obtained data are presented in Table 1. As shown, among all the amino acids, glutamic acid has the highest median value and, as a result, the highest confidence interval values. On the contrary, the lowest median value and confidence intervals were seen in arginine. The highest and the lowest values of SD were observed in glycine and citrulline, respectively. Glutamic acid also showed the lowest CV value, while the highest value was observed in tyrosine. In terms of mean values, the maximum mean value was seen in glutamic acid, while citrulline showed the lowest mean value.

Table 1. The summary of the results. The median, mean, SD, confidence intervals and CV for amino acids were calculated.

Analyte	Median	mean	SD	Confidence intervals (2.5% - 97.5%)		CV
				Lower	Upper	
Alanin	215.00	224.03	65.86	97.00	349.72	0.29
Aspartic Acid	40.30	42.08	13.54	16.17	67.68	0.32
Glutamic Acid	296.00	300.82	63.34	178.94	421.31	0.21
Arginine	9.90	10.85	5.51	0.43	21.13	0.51
Citrulline	10.20	10.81	3.67	4.12	17.36	0.34
Glycine	262.00	270.76	66.02	142.65	397.06	0.24
Leucine	92.10	95.85	23.52	50.27	140.99	0.25
Methionine	18.40	18.77	4.09	10.88	26.57	0.22
Ornithine	85.60	88.93	25.46	39.49	137.79	0.29
Phenylalanine	37.70	38.66	8.35	22.62	54.57	0.22
Proline	143.00	150.70	44.78	63.77	236.53	0.30
Tyrosine	83.30	88.46	32.58	25.79	150.58	0.37
Valine	82.00	84.52	20.77	44.64	123.96	0.25

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Investigating the normality of data distribution

Next, the normality of the data distribution was investigated. For this purpose, the observed values were compared to the expected values on the graphs, and the normality was determined using statistical tests. A normal probability plot is a helpful tool for the normality assessment of the data distribution. It is a quantile-quantile plot (QQ plot) of the standardized obtained data against a standard normal distribution pattern. The correlation and similarity between the sample data and standard normal data indicate the normality of the distribution in the obtained data. There are multiple methods available to assess the normality of continuous data, of which two are the most popular and widely used methods: the Shapiro–Wilk test and the Kolmogorov–Smirnov test. The Shapiro–Wilk test is a more reliable method for small sample sizes (<50 samples) even though it is also used for larger sample numbers. However, the Kolmogorov–Smirnov test is used for large sample sizes (≥50 samples). For both of these tests, the null hypothesis states that data are taken from a population with a normal distribution. When the p-value ≤0.05, the null hypothesis is rejected, indicating that the data are not normally distributed (23). All the measured amino acids in our study showed normal distribution patterns (Fig. 1).

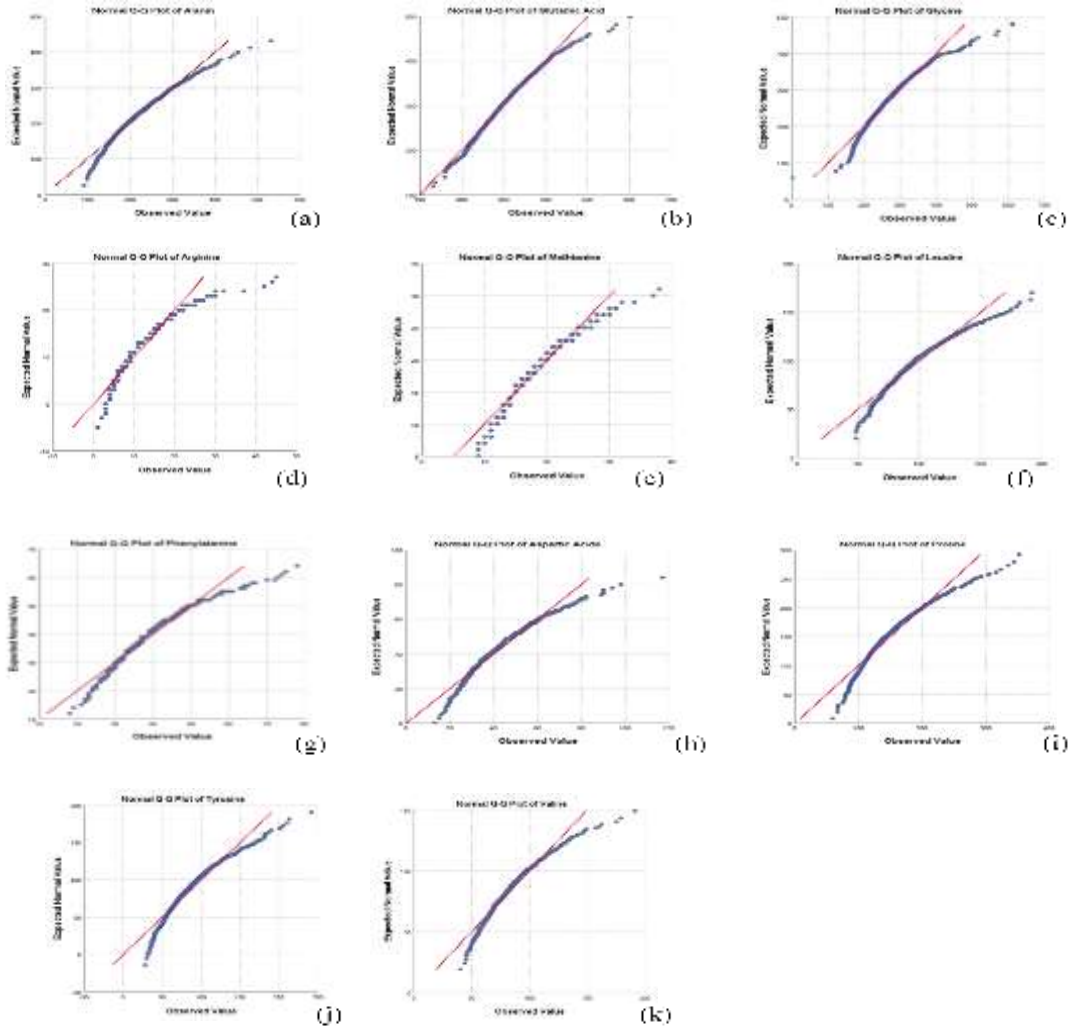

Fig. 1. Normal distribution patterns of all the measured amino acids in our study. All the measured amino acids in our study showed normal distribution patterns. a: Alanine, b: Glutamic Acid, c: Glycine, d: Arginine, e: Methionine, f: Leucine, g: Phenylalanine, h: Aspartic Acid, i: Proline, j: Tyrosine, k: Valine).

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Outliers identification

In the next step, the outlier values for each metabolite were determined. Outlier values are the distinct and separate values from the main pattern of the data or the graph. Values outside of the interval Mean ± 5 SD zone were considered outliers and were removed to stabilize subsequent regression analysis. As shown in Table 2, in our study, glutamic acid had the largest number of outliers, and no outlier values were observed in 5 amino acids, including leucine, methionine, ornithine, phenylalanine, and tyrosine (Table 2).

Table 2. The outlier values obtained for each analyte.

Analyte	Outlier value
Alanine	435, 407, 404, 435, 406, 393, 453, 532, 453, 401, 443, 482, 407, 393, 414
Aspartic Acid	15.7, 13.1, 16.5
Glutamic Acid	439, 461, 504, 551, 601, 448, 496, 462, 427, 483, 457, 451,440, 469, 433, 441, 443, 560, 568, 459, 498, 485, 448, 432, 475, 426, 435, 435
Arginine	44.3, 42.9, 45.6
Citrulline	37.2, 3.95, 44, 3.98, 3.98
Glycine	611
Leucine	
Methionine	
Ornithine	
Phenylalanine	
Proline	323, 59, 316, 323, 335, 352, 344,
Tyrosine	
Valine	179, 191

Reference intervals

Reference intervals are the most reliable tool for supporting the interpretations made on a numerical pathology report. In general, reference intervals indicate the range that contains measured values of a variable in healthy subjects. The reference limit is a value that a particular portion of reference values are less than or equal to it. The obtained data were analyzed and calculated at 0.5%, 99.5%, 1%, and 99% percentiles for the standards and samples for all the amino acids. By comparing the standard values (Table 3) with the obtained data (Table 4), the upper and lower values were calculated. The upper limit of reference intervals is the 99% percentile of healthy individuals, and the 1% percentile of normal subjects is considered the lower limit of reference intervals.

Low cut-off value = median – X SD.

The X value is obtained from Table 3. If an amino acid lacks the X value in the table, the 99.5% and the 0.5% percentiles were considered the high cut-off and the low cut-off value, consequently. The calculated cut-off values for each amino acid are presented in Table 5. As revealed, glutamic acid showed the highest values for either the upper limit (639.395) or lower limit (217.712), while the lowest values of the lower and upper limits were observed in methionine (3.053-40.590) (Table 5).

There are two widely accepted methods to determine reference intervals based on the normality

of the data. We calculate reference intervals according to either method as follows. According to the established methods in clinical laboratories, reference values and cut-off value calculation for each amino acid need

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various parameters, including mean, median, and SD, which are calculated by Excel. Next, the percentiles, including 0.5, 1, 99, and 99.5%, were also calculated using Excel. The 99 and 1 percentile values were considered the highest and lowest values, respectively, in healthy infants. Next, the following equations were employed to calculate the cut-off values for upper and lower limits.

High cut-off value = median + X SD.

Table 3. The data obtained in the R4S study.

Marker	SD LOW= factor X	R4S low CO range		R4S Normal Range			R4S High CO Range		SD HIGH= factor x
		low	High	1%ile	50%ile	99%ile	Low	High	
Ala				117.00	233.00	507.00	507.00	700.00	5.57
Arg	1.05	2.00	5.00	2.30	8.70	32.00	32.00	40.00	4.90
Asp				0.04	0.19	0.66	0.55	0.90	5.33
Cit	1.69	4.00	5.00	6.00	12.00	28.00	28.00	40.00	5.92
Glu				158.00	294.00	551.00	300.00	400.00	1.25
Gln				24.00	50.00	117.00	117.00	150.00	5.00
Gly				185.00	348.00	767.00	500.00	700.00	2.81
Met	1.58	9.80	11.00	11.00	21.00	44.00	44.00	48.00	3.81
Phe				33.00	54.00	97.00	97.00	135.00	5.89
Suac				0.21	0.66	1.40	1.40	7.50	26.74
Tyr				34.00	80.00	207.00	207.00	225.00	3.93
Val				57.00	103.00	212.00	180.00	220.00	3.51
Xle				64.00	115.00	235.00	235.00	250.00	3.95

Table 4. The low and high values of amino acid in samples.

Analyte Name	0.5%ile- Low PCO	99.5%ile- High PCO	1%ile	99%ile
Alanine		435.320	112.840	407.000
Aspartic Acid		90.212	19.892	80.744
Glutamic Acid		498.240	164.600	469.480
Arginine		30.664	3.410	27.656
Citrulline		24.312	5.144	21.116
Glycine		500.240	165.840	480.120
Leucine	63.760		56.320	168.160
Methionine		31.636	11.292	30.240
Ornithine	54.240		40.028	165.080
Phenylalanine		70.108	23.884	64.924
Proline	93.360		79.392	286.320
Tyrosine		200.120	36.400	182.240
Valine		157.120	49.468	145.160

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Table 5. The upper and lower limit values calculated according to method 1 (parametric).

Analyte	Lower limit	Upper limit	99%ile	1%ile	99.5%ile- High PCO	0.5%ile- Low PCO
Alanine	144.100	574.100	407.000	112.840		
Aspartic Acid	29.842	113.396	80.744	19.892		
Glutamic Acid	217.712	639.395	469.480	164.600		
Arginine	15.979	39.318	27.656	3.410		
Citrulline	9.802	29.019	21.116	5.144		
Glycine	80.011	619.757	480.120	165.840		
Leucine					175.160	63.760
Methionine	3.053	40.590	30.240	11.292		
Ornithine					177.080	54.240
Phenylalanine	10.498	82.890	64.924	23.884		
Proline					316.280	93.360
Tyrosine	42.008	260.415	182.240	36.400		
Valine	10.972	194.714	145.160	49.468		

Reference intervals calculated by method 2

This method calculates the reference intervals in a dataset using non-parametric tests and strategies. The 2.5th and 97.5th percentiles were calculated according to CLSI EP-28-A3c guidelines. To determine and validate the results' validity, a confidence interval value close to each reference interval limit is calculated and employed as a metric. Then, outlier values are identified. Subsequently, the calculation method was validated through bootstrapping (23). The findings are presented in Table 6.

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Table 6. The upper and lower limit values calculated according to method 2 (non-parametric).

No	Analyte Name	Reference and Control Internal Limits		
1	**Alanine**	Ref _Int	Lower Ref Limit	Upper Ref Limit (97.5%)
			121.85	370.00
		Conf Int	Lower Ref -Upper	Low Ref Upper Limit
			117-127	356-390
2	**Aspartic Acid**	Ref _Int	Lower Ref Limit	Upper Ref Limit (97.5%)
			21.985	74.300
		Conf Int	Lower Ref -Upper	Low Ref Upper Limit
			20.6-23.2	70.9-77.5
3	**Glutamic Acid**	Ref _Int	Lower Ref Limit	Upper Ref Limit (97.5%)
			190	435
		Conf Int	Lower Ref -Upper	Low Ref Upper Limit
			175-204	416-451
4	**Arginine**	Ref _Int	Lower Ref Limit	Upper Ref Limit (97.5%)
			3.7785	23.2150
		Conf Int	Lower Ref -Upper	Low Ref Upper Limit
			3.61-4.12	21.60-25.70
5	**Citrulline**	Ref _Int	Lower Ref Limit	Upper Ref Limit (97.5%)
			5.9385	19.2150
		Conf Int	Lower Ref -Upper	Low Ref Upper Limit
			5.61-6.16	17.50-20.30
6	**Glycine**	Ref _Int	Lower Ref Limit	Upper Ref Limit (97.5%)
			170.0	413.7
		Conf Int	Lower Ref -Upper	Low Ref Upper Limit
			169-175	399-464
7	**Leucine**	Ref _Int	Lower Ref Limit	Upper Ref Limit (97.5%)
			60.27	152.00
		Conf Int	Lower Ref -Upper	Low Ref Upper Limit
			59.3-60.9	147.0-158.0
8	**Methionine**	Ref _Int	Lower Ref Limit	Upper Ref Limit (97.5%)
			12.4	28.2
		Conf Int	Lower Ref -Upper	Low Ref Upper Limit
			12.0-12.7	27.3-29.0
9	**Ornithine**	Ref _Int	Lower Ref Limit	Upper Ref Limit (97.5%)
			49.855	148.000
		Conf Int	Lower Ref -Upper	Low Ref Upper Limit
			45.5-51.7	141.0-155.0
10	**Phenylalanine**	Ref _Int	Lower Ref Limit	Upper Ref Limit (97.5%)
			25.685	58.215
		Conf Int	Lower Ref -Upper	Low Ref Upper Limit
			24.8-26.4	56.3-61.2
11	**Proline**	Ref _Int	Lower Ref Limit	Upper Ref Limit (97.5%)
			85.60	262.15
		Conf Int	Lower Ref -Upper	Low Ref Upper Limit
			84.5 -88.4	247.0-272.0
12	**Tyrosine**	Ref _Int	Lower Ref Limit	Upper Ref Limit (97.5%)
			40.285	170.150
		Conf Int	Lower Ref -Upper	Low Ref Upper Limit
			38.7-42.0	162.0-180.0
13	**Valine**	Ref _Int	Lower Ref Limit	Upper Ref Limit (97.5%)
			52.655	132.000
		Conf Int	Lower Ref -Upper	Low Ref Upper Limit
			50.9-55.0	127.0-136.0

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Discussion

Inborn errors of metabolism (IEMs) constitute a broad spectrum of disorders with diverse clinical presentations and symptoms, posing challenges to accurate diagnosis for physicians. The measurement of amino acid concentrations in circulation is a dependable method for diagnosing IEMs. Given the varied symptoms and the potential for severe clinical outcomes, including irreversible nervous system damage, morbidity, and mortality, early diagnosis and appropriate therapeutic interventions are crucial for patient survival and quality of life. In recent decades, significant advancements in screening and monitoring IEMs globally have been achieved primarily through diagnostic technologies, particularly LC-MS/MS. Many countries have implemented newborn screening (NBS) programs using LC-MS/MS on dried blood spots (DBS), leading to the diagnosis of various metabolic disorders and IEM-related diseases (24). Therefore, this study aimed to establish cut-off values for amino acids by analyzing DBS samples obtained from healthy subjects in Mashhad, Northeast Iran.

Today, LC-MS/MS technology is widely employed for the screening of IEMs. It is a powerful technology that is able to simultaneously detect multiple metabolic disorders in a small amount of sample using a single analytical high-throughput technique (25). Moreover, LC-MS/MS technologies have some advantages over other methods, including ease of sampling and sample storage, rapidity, high sensitivity and specificity, as well as the stability of internal standards, which consequently increase the sensitivity and validity of the results. Despite the fact that the LC-MS/MS method is able to detect the majority of metabolic disorders, a meticulously established cut-off value is critical to ensure correct detection. A valid cut-off value not only facilitates the diagnosis process but also reduces the incidence of false positive or false negative cases (26-28). The global cut-off values for IEM screening were determined locally in multiple studies and globally in a worldwide project with the collaboration of many countries. In the R4S project, 25-30 million healthy neonates and newborns participated, and besides cut-off determination, 10742 cases were also diagnosed with IEMs (9). It is noteworthy that multiple factors influence the cut-off values, including the ethnicity of the target population, genetic background, the method used for analysis, and the technology of the instruments employed (28, 29). Regarding the factors that influence the results, previous data showed that variations in the concentrations of blood amino acids are not sex-dependent in the neonatal period. However, some studies indicated that gender might affect the amino acid levels in older children and adults (30-33). Therefore, in this study and other studies on neonates, the cut-off values are adjusted and calculated according to the age of the participants.

In the present study, we measured the concentrations of amino acids in the DBS of 1005 healthy neonates aged 2-7 days and calculated the reference intervals for amino acids in DBS samples in Mashhad, Iran. When using the Kolmogorov-Smirnov test, median values for all amino acids were significantly different from previous reports. Previously, the reference intervals for IEM screening were established in different regions in Asia. However, since IEMs are inherited metabolic disorders and genetic background and ethnicity are directly related to the incidence rates, reference interval establishment must be regional.

Formerly, reference intervals for amino acids have been established globally and in different regions, including North America, Asia, and Europe, using different methods and approaches. For instance, Svasti et al. measured the levels of free plasma amino acids in the Thai children population, categorizing specimens into age groups (0–6 months, 6-12 months, 1-3 years, 3-6 years, and 6-12 years) and measuring amino acid concentrations with reverse-phase HPLC and pre-column derivatization by phenylisothiocyanate(34). Similarly, in a more recent study in Thailand,

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Uaariyapanichkul et al. measured the reference intervals for amino acids in the Thai pediatric population using LC-MS/MS, which differed from the previous study that used HPLC.

In a worldwide collaborative project involving 45 countries, the plasma levels of amino acids were measured in a study population of 25-30 million, establishing global cut-off values for amino acids and acylcarnitines. However, the values found in this study showed slight differences from previous regional reports. Sarker et al. also calculated age-specific cut-off values for amino acids in Bangladesh. In this study, 570 subjects were enrolled and divided into three groups based on age: newborns aged 1-7 days, the second group 8 days-7 years, and the last group consisting of individuals 8-17 years. Their results led to the establishment of cut-off values for screening Bangladesh's population and also revealed the most dominant kinds of IEMs in Bangladesh (11). Despite some similarities between our study and the former study, our results revealed different normal ranges compared to the previous study, with differences in sample size and ethnicity between the two studies. Similarly, a recent investigation conducted by Bairova et al. in Irkutsk, the Asian region of Russia, reported the reference intervals for amino acids in the northern Asian population. According to their results, branched-chain amino acids, including Valine, Leucine, and Isoleucine, showed higher concentrations in the northern Asian population compared to other populations. There was also a slight difference in reference interval values for Phenylalanine, Tyrosine, Citrulline, Alanine, Ornithine, and Proline compared to other nations. Arginine, Aspartic Acid, and Glutamic Acid had higher neonatal blood concentrations in northern Asia compared to other regions (35).

In conclusion, we established reference interval values for 13 amino acids in Mashhad, Northeast Iran, using the LC-MS/MS method.

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Conflicts of interest

All authors declare they have no conflicts of interest.

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