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Reference Interval for Plasma Free Amino Acids in Iranian Children Using Reverse Phase High Performance Liquid Chromatography

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Abstract

Background: Brain damage and mental retardation in children can be caused by either excess or depletion of certain amino acids. Up to now there is no reliable reference interval for free plasma amino acid in Iranian children.
Objectives: The aim of this study was to provide the reference intervals of free plasma amino acid in healthy Iranian children using high performance liquid chromatography (HPLC) as an accurate method.
Methods: 133 children referred to Children's Medical Center, Tehran, Iran for the annual checkup entered the study after filling out the informed consent form. Demographic data and blood samples after overnight fasting were collected. Plasma amino acid concentrations were determined by reverse phase HPLC (RP-HPLC) equipped with a fluorescence detector after precolumn derivatization of amino acids with o-phthalaldehyde (OPA).
Results: Female children exhibited significantly higher plasma concentrations of taurine and lysine than male children. Other 20 amino acid values were not significantly different between two gender groups. Positive correlation was obsereved between age and taurine, leucine and lysine.
Conclusions: Our data provided reference intervals of free plasma amino acids in healthy Iranian children. We believe our data can be used as a guideline for pediatricians to identify the significantly deviated amino acid values in children with medical issues such as genetic disorders and some acquired illnesses. Our data is also applicable for further investigations in the field of family medicine.

Keywords: Amino Acid, High Performance Liquid Chromatography, Reference Intervals, Children

1. Background

Excess or depletion of certain amino acids will result in severe illnesses (e.g. Phenylketonuria and Maple Syrup Urine Disease) which are usually accompanied by brain damage and mental retardation (1-3). The amino acid baseline concentrations are used in assessment of nutritional status and creating amino acid level profiles (4-6). Recent studies have emphasized the importance of these nutritional profiles in prognosis of malnourished patients with chronic renal and liver diseases (7, 8). In the recent years, obtaining the nutritional profile in patients undergoing dialysis for the first time or transplant surgery, is considered as a major factor in prognosis, survival rate, mortality and morbidity (4, 8, 9).

Defining the reference interval of amino acids is important for diagnostic and prognostic purposes. Free plasma amino acid concentrations can differ in normal individuals over a broad range of variables like diet, time of the day, gender, obesity, stress, hormones like testosterone and age. Among the identified variables, age and gender are the two main physiologic factors affecting plasma amino acids values. Significant differences between male and female and between old and young adults have been reported by several studies (4, 10-13).

Although free plasma amino acid values in children are documented in medical textbooks and journals (12, 14, 15) no equivalent study has been conducted in the Iranian child population.

2. Objectives

To determine the normal free plasma values which are the primary requirement for physicians to apply to their practices, we have measured free plasma concentration of 22 amino acids in healthy Iranian children aged 2 to 8 years, analyzed by o-phthalaldehyde derivative high performance liquid chromatography (OPA-HPLC) method.

3. Methods

A total of 133 Iranian children (54 females and 79 males, 2 - 8 years old) who were referred to Children's Medical Center Hospital, Tehran, Iran for their annual check-up from July 2011 to June 2012 were recruited into the project after filling out the informed consent form by parents. The participants fulfilled the requirements of the inclusion criteria. The study size was reckoned according to CLSI guideline (C28-A2) recommendation for establishment of reference interval (16).

The inclusion criteria were as follows: a) no history of metabolic disorders, b) no history of liver or kidney diseases, c) normal development and growth d) being on a regular diet and not consuming infant formula), and finally d) no evidence of malnutrition.

Overnight fasting blood samples from antecubital vein were collected in a heparinized syringe and immediately separated by centrifugation at 3000 rpm for 15 minutes. 200 μL of plasma was mixed with 50 μL internal standard homoserine, afterwards ice-cold methanol (800 μL) was applied to the mixture while vortexing, then kept in 4°C for 5 minutes.

The tubes were centrifuged at 4,000 rpm for 5 minutes and supernatant was collected. 250 μL of supernatant was mixed with 100 μL of borate buffer followed by 50 μL of OPA/2ME reagent (Tube A). 200 μL of mobile phase and 25 μL of hydrochloric acid (HCl) was added to 50 μL of Tube A solution and injected to HPLC system (Knauer, Germany). Separation of derivatives of amino acids was performed by reversed-phase HPLC chromatography on a C18 column using a gradient of 0.05 M acetate buffer and methanol, pH 7.02 with a flow rate of 1.0 mL per minute.

HPLC was equipped with a fluorescence detector operating at excitation wavelength of 330 nm and emission wavelength of 450 nm.

Amino acids including alanine, arginine, asparagine, aspartic acid, citrulline, α-aminobutyric acid, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, serine, taurine, threonine, tryptophane, tyrosine and valine were quantified using appropriate calibrators (Clin Cal, RECIPE, Gm pH, Germany).

Plasma amino acid concentrations were expressed as mean, 95 per cent confidence interval and scored at 5th, 25th, 75th and 95th percentiles. Amino acid concentrations were compared according to age and gender by analysis of variance (ANOVA) and t-test, respectively. IBM SPSS Statistics 17.0 software was used for statistical analysis. P value $<$ 0.05 was considered statistically significant. The study was performed in agreement with Helsinki declaration and was conducted in accordance with considerations recommended by local ethics review committee of Tehran University of Medical Sciences (ethics code: 90-02-30-12879).

4. Results

The mean age of 133 children (54 female and 79 male) was 4.2 years (ranged from 2 to 8 years). Mean of each measured free plasma amino acid concentration, 95 per cent Confidence Interval and the scores of 5th, 25th, 75th and 95th percentiles are presented in Tables 1 and 2, respectively.

A significant correlation between age and taurine (r = 0.185, P = 0.033), leucine (r = 0.184, P = 0.034) and lysine (r = 0.175, P = 0.043) was observed.

5. Discussion

This study presents the physiologic values of 22 amino acids in Iranian children aged 2 to 8 years and their distribution as the scores of 5th, 25th, 75th and 95th percentiles. Since maximum and minimum values could have unusually high or unusually low values, we preferred to use the 95 per cent confidence interval to describe our data. These findings are intended to act as a reference for pediatricians to identify the significantly deviated amino acid values in Iranian children with medical issues such as genetic disorders and some acquired illnesses.

In the present study we used a RP-HPLC method involving pre-column derivatization with o-phthalaldehyde (OPA) to analyze the free plasma amino acid concentrations. Although simplicity and reproductivity of ion exchange chromatography (IEC) in combination with post-column ninhydrin make it the reference method for measuring the free plasma amino acids in clinical laboratories (4, 17-19), its long analytical run time made the OPA-HPLC a useful alternative method for analyzing the amino acids in physiological fluids because of OPA-HPLC's higher sensitivity and speed, both of which are very important for newborn screening (18).

The data of the present study demonstrated two additional findings. First, the plasma concentration of taurine and lysine was significantly higher in female children than in male ones and the other 20 measured amino acids were not significantly different between the two gender groups. Our results were in consistency with the report of Gregory

Table 1. Mean Concentration and 95% Confidence Interval of Amino Acids in Healthy Children

Amino Acid	Mean, μMol/L	Confidence Interval 95%	Amino Acid	Mean, μMol/L	Confidence Interval 95%
Alanine	376.39	358.36 - 402.59	Leucine	115.379	108.48 - 122.24
Arginine	71.813	67.10 - 76.15	Lysine	131.099	123.46 - 136.07
Asparagine	55.4815	53.25 - 57.99	Methionine	26.44	24.49 - 28.75
Aspartic acid	5.92	5.34 - 6.35	Ornithine	62.02	58.42 - 64.83
Citrulline	26.52	24.97 - 28.04	Phenylalanine	61.40	59.00 - 64.49
γ-Aminobutyric acid	23.10	21.53 - 24.25	Serine	139.68	134.20 - 144.07
Glutamic acid	59.71	54.21 - 65.27	Taurine	77.11	68.11 - 82.61
Glutamine	552.12	534.19 - 566.89	Threonine	121.18	113.61 - 127.00
Glycine	258.15	245.97 - 270.03	Tryptophane	50.15	47.20 - 53.22
Histidine	91.56	86.71 -95.14	Tyrosine	67.01	63.26 - 70.57
Isoleucine	66.72	62.72 - 70.90	Valine	227.48	216.76 - 238.17

Table 2. Values of Amino Acids for the Scores of 5th, 25th, 75th and 95th Percentiles

Amino Acid	Percentile, μMol/L			
	5	25	75	95
Alanine	184.06	284.50	466.20	581.16
Arginine	37.02	55.70	82.40	134.08
Asparagine	35.82	45.10	64.70	79.52
Aspartic acid	2.36	3.70	7.30	11.58
Citrulline	13.70	20.20	32.60	42.10
γ-aminobutyric acid	13.22	17.60	25.40	41.78
Glutamic acid	22.44	35.87	76.77	127.95
Glutamine	372.88	495.00	628.80	687.00
Glycine	164.62	208.80	308.40	408.40
Histidine	56.340	73.90	108.40	135.44
Isoleucine	37.020	50.10	77.00	114.42
Leucine	65.22	88.90	134.30	196.50
Lysine	85.26	102.10	148.80	202.34
Methionine	11.72	18.00	31.70	50.16
Ornithine	33.02	48.70	73.40	94.88
Phenylalanine	38.91	50.75	71.13	97.87
Serine	95.34	118.00	154.60	194.00
Taurine	27.64	44.40	98.80	178.72
Threonine	64.94	93.10	140.20	197.00
Tryptophane	15.62	40.10	61.00	79.70
Tyrosine	38.72	51.90	82.00	110.52
Valine	144.40	181.20	264.10	346.86

et al that the effect of sex was not apparent in children, although they found significant difference for 5 amino acids including isoleucine, leucine, methionine, phenylalanine, and tyrosine in adolescents (20). Their values were significantly lower in adult females than in males. In a study conducted by Yamamoto et al. a link between sex and concentration of most of the amino acids especially branched chain amino acids was reported (21). A few other studies have also identified lower concentrations of branched-chain amino acids in older females (10, 11). Since several studies found sex-dependent amino acid values in adolescents and no significant differences in preadolescent children (10, 22, 23), it is believed that these gender-related differences occur after adolescence.

Recent studies on amino acid concentrations have described a relation between diet and amino acid values. Milson et al studied on fasting and postprandial plasma amino acid concentration and showed a gender-related difference in fasting subjects (24). They have reported that fasting female subjects had lower values in several amino acids than fasting male ones, but this effect was insignificant in postprandial subjects. Therefore, dietary protein can induce a significant degree of variation in amino acid values throughout a day. Another study, conducted by Rana et al. showed that protein intake is significantly lower in vegan diets particularly taurine which was absent in vegan diets (25). In comparison to these data, we suggest that the lower amount of taurine and lysine could be a result of a daily diet variation in children. It needs a study with more cases and precise diet control.

Our second finding identified a significant relation between age and lysine, leucine and taurine values. Our results to some extent, were consistent with previous studies which are suggesting an increase in amino acid levels in growing children probably caused by increasing muscle mass (26). Lepage et al. demonstrated a unique pattern of age-specific distribution for each amino acid in a healthy pediatric population (27). In their study, alanine, arginine, asparagine, methionine, ornithine, phenylalanine, proline, threonine and tyrosine were decreased in the first years of life, and then increased steadily up to 18 years of age. An Initial reduction followed by stable concentration was observed in taurine and serine. Aspartic acid and glutamic acid had decreasing trend in all ages. Citrulline demonstrated a two-step increment at 0 to 3 years of age and 13 to 15 years. Cystine, glutamine, glycine, histidine, isoleucine, leucine, lysine, tryptophan, and valine showed increasing values throughout 0 to 18 years of age. Several other studies (20, 28, 29) have also reported a specific pattern of age-dependent amino acid concentrations with slight differences to Lepage et al.'s study. It seems that children under one year need specific reference value. Cruz et al. in their study validated the HPLC method for determination plasma amino acids and established reference interval for Brazilian population (30). They recommended that each laboratory should establish its own reference interval due to variation in methodology and analytical techniques used in published data. The main limitation of our study, as that of Cruz et al.'s, was absence of specimens from children under 2 years old. That was because their parents refused to give permission for blood collection from their babies for our study.

This study provided reference intervals of free plasma amino acids in healthy children. We believe our data can be used as a guideline for investigations in the field of family medicine and in the practice of pediatricians. We have also reported an significant difference between age and 3 of amino acid levels and also a significant higher concentration of taurine and lysine in female children whereas other amino acids were not significantly different, but it is more ideal to establish further follow up studies and include more affecting factors such as, body mass index, and diet.

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