

# Amino acid reference intervals by high performance liquid chromatography in plasma sample of Brazilian children

## *Valores de referência de aminoácidos por cromatografia líquida de alta eficiência em amostras de plasma de crianças brasileiras*

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### ABSTRACT

**Introduction:** The high performance liquid chromatography is a technique used for quantification of amino acids in plasma. The definition of reference intervals in the population is very important for the diagnosis and monitoring of individuals with amino acid disorders. **Objectives:** The objectives of this study were to validate a method for amino acids quantification and define reference intervals in Brazilian children. **Results:** Good chromatographic separation was achieved using C18 solid-core column. The method showed satisfactory linearity, limits of detection and quantification, precision and accuracy. The reference ranges for aspartate, glutamate, asparagine, histidine, serine, glutamine, arginine, tyrosine, alanine, tryptophan, methionine, valine, phenylalanine, isoleucine and leucine were defined in a group of 60 healthy individuals aged 2 to 14 years. **Conclusion:** The presented technique can be applied in clinical practice. Each laboratory should preferably establish its own reference intervals. If that is not possible, it is recommended the use of the reference intervals described in this study for the diagnosis and monitoring of Brazilian children in this age group.

**Key words:** liquid chromatography; inborn errors of metabolism; amino acids; reference values.

### INTRODUCTION

Inborn errors of metabolism constitutes a diverse group of individually rare disorders, that group correspond to 10% of the genetic diseases described<sup>(1)</sup>. They all occur due to enzyme defects that cause disruption of specific metabolic pathways<sup>(2)</sup>. Within this group, the amino acids disorders stand out as serious diseases, whose symptoms are caused by acute or chronic poisoning by the accumulation of amino acids and other toxic metabolites, which might damage the brain, kidneys and liver<sup>(1,3)</sup>. Their relative rarity and nonspecific presentation may contribute to the delay or the difficulty to diagnosis, which leads, sometimes, to irreparable consequences on child development<sup>(4-6)</sup>.

Thereby, quantitative analysis of amino acids in blood and urine are tests especially indicated for patients suspected of amino acid disorders and organic acidemia. These exams can also be

applied in the management of patients with these diseases that make use of dietary formulas restricted in amino acids and also for therapeutic decisions during decompensation and crisis<sup>(7, 8)</sup>. A decrease or mild elevation of amino acids level can only be detected in blood, and morning fasting blood specimens are preferred. High performance liquid chromatography (HPLC) with derivatization is widely used for the diagnosis of most amino acid disorders.

Amino acids levels must be cautiously interpreted, since the levels of these substances in the blood are influenced by a number of factors, such as age, physiological changes, nutritional status, illnesses, medications and toxins. Furthermore, there are no well-established reference intervals in our population, and data from international literature are traditionally used<sup>(9-12)</sup>. The aim of this study was, therefore, to validate a technique of measurement of amino acids in plasma using HPLC with fluorometric detector and solid-core column, as well as define reference ranges for serum amino acids for the pediatric group from the population studied.

## MATERIAL AND METHOD

### Equipment

The equipment used was the HPLC Alliance HT Waters® 2695 (Milford, USA) equipped with quaternary pump, thermostat, automatic injector, degasser and fluorometric detector multivariable model 2475. The system was controlled by Empower 2.0 software.

### Standards, chemicals and reagents

The standards and chemicals utilized in the methodology were purchased from Sigma Aldrich® (St. Louis, USA) and included: L-aspartic acid, L-glutamic acid, L-asparagine, L-histidine, L-serine, L-glutamine, L-arginine, L-glycine, L-threonine, L-tyrosine, L-alanine, L-tryptophan, L-methionine, L-valine, L-phenylalanine, L-isoleucine, L-leucine, mercaptoethanol (analytical grade) and ortho-phthalaldehyde (OPA) (analytical grade). The others chemicals and reagents utilized were from Merck® (Darmstadt, Germany) and include: acetonitrile (HPLC grade), formic acid (analytical grade), methanol (HPLC grade), sodium hydroxide (analytical grade), ethanol (analytical grade), sodium phosphate monobasic dihydrate (analytical grade), sodium phosphate dibasic (analytical grade), orthophosphoric acid (analytical grade) and boric acid (analytical grade). The deionized water used was from the water purification system of Millipore® (Millipore, Bedford, MA, USA) with resistivity of 18.2 megaohms.cm-1.

### Solutions

The derivatization solution was formed by OPA (0.02 g) prepared with mercaptoethanol (20 µl), ethanol (100 µl) and 0.4 M borate buffer in alkaline environment (pH 9.5 to 1 ml). Solutions of 20% formic acid, 20% methanol and 50% acetonitrile were utilized to prepare stock solutions of each amino acid (Table 1). The intermediary solutions and solutions used were prepared with reagent grade water.

TABLE 1 – Solubility of the amino acids analyzed

Standards	Solvent	Standards	Solvent
Aspartic acid	Formic acid 20%	Alanine	Reagent grade water
Glutamic acid	Reagent grade water	Tryptophan	Acetonitrile 50%
Asparagine	Methanol 20%	Methionine	Acetonitrile 50%
Serine	Methanol 20%	Valine	Acetonitrile 50%
Histidine	Methanol 20%	Phenylalanine	Acetonitrile 50%
Glutamine	Methanol 20%	Isoleucine	Reagent grade water
Arginine	Methanol 20%	Leucine	Reagent grade water
Tyrosine	Formic acid 20%		

### Preparation of mobile phase and chromatographic conditions

The gradient mode (Table 2) was used for better separation of amino acids and two mobile phases were prepared. The mobile phase A composition was phosphate buffer, methanol and water (57/33/30% v/v) with pH 6.5 and mobile phase B composition was methanol and phosphate buffer (47/53 v/v%) with pH 8.5. The wash solution was constituted by methanol 10%. The solid-core column AccuCORE C18 (150 mm × 4.6 mm, particle size 2.6 mM) of Thermo Scientific® (Waltham, USA) was used to achieve separation of the amino acids. The wavelength of the detector was  $\lambda_{\text{ex}} = 330 \text{ nm}$  and  $\lambda_{\text{em}} = 418 \text{ nm}$ .

### Validation methods

After method optimization, statistical tools were applied using the software Statistical Package of the Social Sciences 12.0® (SPSS).

### Linearity

The concentrations of aspartate (100, 200, 300 and 400 nmol/ml), glutamate (150, 600, 1050 and 1500 nmol/ml), asparagine (150, 600, 1050 and 1500 nmol/ml), histidine (100, 250, 400 and 550 nmol/ml), serine (150, 600, 1050 and 1500 nmol/ml), glutamine (150, 600, 1050 and 1500 nmol/ml), arginine (100, 250, 400 and 550 nmol/ml), tyrosine (150, 600, 1050 and 1500 nmol/ml), alanine (150, 400, 750 and 1050 nmol/ml), tryptophan (150, 600, 1050 and 1500 nmol/ml), methionine (100, 200, 300 and 400 nmol/ml), valine (150, 600, 1050 and 1500 nmol/ml), phenylalanine (150, 600, 1050 and 1500 nmol/ml), isoleucine (150, 600, 1050 and 1500 nmol/ml) and leucine (150, 600, 1050 and 1500 nmol/ml) were prepared for linearity study. These solutions were processed as described in the topic “Sample collection and processing”, injected in triplicate on the same day of analysis.

The linearity was studied in four concentration levels. The analytical curves and the coefficient of determination ( $r^2$ ) were obtained and the method was considered linear when  $r^2$  was greater than 0.99.

TABLE 2 – Mobile phase gradient established by amino acid analysis

Time (min)	Flush	% Mobile phase A	% Mobile phase B
0	0.2	100	0
25	0.2	100	0
26	0.2	32	68
61	0.3	0	100
80	0.3	0	100
85	0.3	100	0

### *Limit of detection (LOD) and quantification (LQ)*

To evaluate the LOD and LQ of the method, 10 independent replicates were prepared from the white plasma pool. The solutions were processed as described in the topic "Sample collection and processing". A signal-to-noise ratio of 3:1 was used to determine the LOD and a ratio of 10:1 was used to determine the LQ of the method.

### *Precision and accuracy*

The different concentrations of aspartate (10, 100 and 350 nmol/ml), glutamate (250, 350 and 1050 nmol/ml), asparagine (25, 250 and 1050 nmol/ml), histidine (100, 150 and 350 nmol/ml), serine (140, 180 and 1050 nmol/ml), glutamine (180, 350 and 1050 nmol/ml), arginine (120, 150 and 400 nmol/ml), tyrosine (80, 180 and 1050 nmol/ml), alanine (350, 550 and 1050 nmol/ml), tryptophan (70, 150 and 400 nmol/ml), methionine (35, 100 and 350 nmol/ml), valine (150, 250 and 1050 nmol/ml), phenylalanine (70, 180 and 1050 nmol/ml), isoleucine (100, 180, and 1050 nmol/ml) and leucine (150, 250 and 1050 nmol/ml) were prepared in triplicate for the study of precision and accuracy. These concentrations were processed as described in the topic "Sample collection and processing".

For precision, the solutions were analyzed by chromatography on the same day of preparation (intra-assay precision) and on three consecutive days (inter-assay precision). The intra-assay and inter-assay precision were expressed by the coefficient of variation. The accuracy of the method was verified by triplicate analysis of each amino acid and the recovery percentage was calculated.

### **Ethical aspects**

This study was approved by the Research Ethics Committee of the Universidade Federal de Minas Gerais (UFMG) under registration number 08169212.2.0000.5149 and all participants and/or guardians signed the informed consent form.

### **Population**

A cross sectional study was carried out with individuals selected in the Hospital das Clínicas of the UFMG, among children from primary and secondary care units linked to the institution. The group was formed by 60 individuals aged between 2 and 14 years, of both genders. The sampling was done considering the exclusion and inclusion criteria and the period of two months available for the study, characterizing a convenience sampling. All children in this age group who attended in central laboratory for blood collection were interviewed for the identification of any exclusion criteria.

### **Inclusion criteria**

Healthy children who attended the Hospital for performing routine checkups were included. All subjects responded to a questionnaire on date of birth, previous diseases, use of medications, fasting time, and presence of vomiting.

### **Exclusion criteria**

Children with history of short stature or insufficient weight gain, who presented fasting or vomiting lasting more than a day or who were in use of interfering substances during the collection period, such as ascorbic acid, aspartame, aspirin, sulfamethoxazole/trimethoprim, glucose, indomethacin, progesterone, testosterone and valproate were excluded. Patient in pre or post-transplant, with liver or kidney disease, type 1 diabetes, sickle cell anemia, leukemia and other cancers, cystic fibrosis, genetic syndromes and dysmorphisms, acquired immunodeficiency syndrome (Aids), syphilis, visceral and hematological alterations under investigation, autoimmune diseases, endocrine and hormonal disorders were also excluded<sup>(13)</sup>. Younger children, especially under one year, were not included in the group, because blood levels of amino acids present greater variation in these children compared with the age group studied<sup>(13,14)</sup>.

### **Sample collection and processing**

The sample collection was performed after fasting of 8-12 hours. For each patient were collected 4 ml of venous blood in a Vacutainer ethylenediaminetetraacetic acid (EDTA) blood collection tube. The sample were centrifuged at 2,000 rpm for 5 minutes to separate plasma and immediately frozen at a temperature of -20°C until the time of analysis, which was carried out in a maximum period of 15 days.

Sample preparation consisted of adding 20 µl of plasma to an Eppendorf and 100 µl of the derivatizing solution previously described. The solution was homogenized with the aid of a pipette and transferred into an insert. After 1 minute, 1 µl of the solution was injected into the chromatograph.

### **Statistical analysis**

For definition of the reference ranges, statistical analysis was performed using the software Medcalc<sup>®</sup>. The detection of probable outliers was tested by Reed's method<sup>(15)</sup>. The reference ranges were calculated using the robust method, after logarithmic transformation when the raw data were not parametric

according to Kolmogorov-Smirnov test<sup>(16)</sup>. For data that did not show parametric distribution, even after transformation, non-parametric methodology was used to calculate the lower limit, which corresponds to 2.5 percentile. The upper limit was calculated by the robust method, according to the method described by Horn and Pesce (2005)<sup>(17)</sup>. Confidence intervals of 90% for the lower and upper limits of each interval were generated, except when the non-parametric method was applied, because  $n < 120$ <sup>(16)</sup>. The Student's *t* test and Wilcoxon test were applied to comparison with the literature.

## RESULTS

### Method validation

After optimization, the run time was 90 minutes and the gradient established allowed a good chromatographic separation of the validated amino acids (**Figure 1**). The amino acids glycine and threonine showed analytical signal amongst amino acids arginine and tyrosine, but did not provide good chromatographic separation and were not included in this validation. Linear responses were found with  $r^2 > 0.99$  for all amino acids (**Table 3**). The values for LOD and LQ are described in Table 3. The results of accuracy, intra and inter-assay obtained in the three different concentrations are described in **Table 4**. The recovery presented values between  $(87.9 \pm 5.6)\%$  and  $(112.3 \pm 6.1)\%$ . The results were reported in **Table 5**.

### Determination of reference intervals

**Figure 2** illustrates the distribution of the control group by gender and age. **Table 6** shows the reference ranges calculated for all amino acids studied.

## DISCUSSION

### Method validation

The sample preparation is simple and fast, but should not be reinjected into the chromatograph since the reaction of amino acids with OPA is rather unstable and these compounds can be rapidly converted into non-fluorescent degradation products<sup>(18)</sup>. The disadvantage of using OPA consists in the instability of the fluorescent product and in its restriction of only reacting with primary amino acids. This is counterbalanced by the fact that its use allows high sensitivity and reproducible analysis<sup>(19)</sup> and

its relatively low cost, making OPA a widely used derivatization reagent for the analysis of amino acid<sup>(18,20)</sup>.

The procedure was considered long for a laboratory routine. That occurs because, although the use of solid core column allowed excellent separation and good durability, when it is introduced into HPLC it requires a lower flow rate for not reaching high pressures.

The amino acids glycine and threonine were not included in the validation since they do not have good chromatographic separation. This can be explained by the fact that the retention time of both amino acids were subjected to the gradient of mobile phases A (32.0%) and B (68.0%).

Under the conditions tested, the gradient, that it is a mixture of mobile phases of different compositions, provided the coelution of threonine and glycine, favored the time for the chromatographic run and did not impair the determination and the reliability of other validated amino acids.

The results of the linearity limits of detection and quantification method were satisfactory.

The greatest variation found in the intra-assay (12.3%) was attributed to aspartate and can be explained by the lower concentration level for this amino acid (10  $\mu\text{mol/l}$ ). Comparing the results obtained in the intra-assay precision with the biological variation<sup>(21)</sup> described in Table 4, it is observed that all variations found in this study were lower than the established biological variation.

In the inter-assay, glutamine showed the greatest variation (15.2%-concentration of 180  $\mu\text{mol/l}$ ). This variation found was even higher than the within-subject biologic variation<sup>(21)</sup> described in Table 4. The variability attributed to glutamine may be connected to the gradient that starts exactly at the retention time of the glutamine, thus influencing the increased variability. Fekkes (1996) assessed the inter-assay precision ( $n = 12$ ) using HPLC and OPA-derivatization, the author also noted that the amino acid glutamine presented the greatest variation (25%) in the study.

For other amino acids, all variations in the inter-assay precision were less than the biological variation contained in Table 4.

### Determination of reference intervals

The number of individuals included in control group is similar to other studies that established reference intervals for plasma amino acids in children, such as Gregory *et al.* (1986)<sup>(9)</sup>, Lepage *et al.* (1997)<sup>(10)</sup> and Strauss *et al.* (2010)<sup>(12)</sup>.

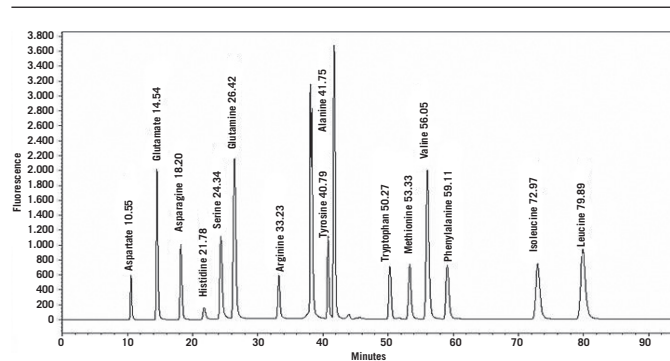


FIGURE 1 – Determination of amino acids in aqueous matrix by HPLC

Aspartate (100 nmol/ml), glutamate (250 nmol/ml), asparagine (25 nmol/ml), histidine (100 nmol/ml), serine (140 nmol/ml), glutamine (350 nmol/ml) arginine (150 nmol/ml), tyrosine (180 nmol/ml), alanine (350 nmol/ml), tryptophan (150 nmol/ml), methionine (100 nmol/ml), valine (250 nmol/ml), phenylalanine (180 nmol/ml), isoleucine (180 nmol/ml) and leucine (250 nmol/ml).

HPLC: high performance liquid chromatography.

TABLE 3 – Linearity, limit of detection and quantification of the method

Compound	Range (nmol/ml)	Linearity (nmol/ml)	r <sup>2a</sup>	LOD <sup>b</sup> (nmol/ml)	LQ <sup>c</sup> (nmol/ml)
Aspartate	100-400	400	0.993	0.67	2
Glutamate	150-1500	1500	0.990	1.67	5
Asparagine	150-1500	1500	0.996	1.67	5
Histidine	100-550	550	0.990	1.67	5
Serine	150-1500	1500	0.995	1.67	5
Glutamine	150-1500	1500	0.991	2.33	7
Arginine	100-550	550	0.991	1.67	5
Tyrosine	150-1500	1500	0.994	1.67	5
Alanine	150-1050	1050	0.990	3.33	10
Tryptophan	150-1500	1500	0.992	1.67	5
Methionine	100-400	400	0.992	1.67	5
Valine	150-1500	1500	0.996	1.67	5
Phenylalanine	150-1500	1500	0.993	3.33	10
Isoleucine	150-1500	1500	0.9903	1.67	5
Leucine	150-1500	1500	0.9943	3.33	10

r<sup>2a</sup>: coefficient of determination; LOD<sup>b</sup>: limit of detection; LQ<sup>c</sup>: limit of quantification.

TABLE 4 – Precision of the method

Compound	Precision	CV <sup>a</sup> (%)			Biological variation (%)	
		Concentration 1	Concentration 2	Concentration 3	CVi <sup>d</sup>	CVg <sup>e</sup>
Aspartate	Intra <sup>b</sup>	12.3	4.84	5.1	31.2	55.1
	Inter <sup>c</sup>	10.6	5.45	11.9		
Glutamate	Intra	4.57	6.13	6.13	46.4	79.9
	Inter	7.1	4.8	4.9		
Asparagine	Intra	4.67	4.81	7.94	12.3	28
	Inter	5.45	6.22	6.92		
Histidine	Intra	7.42	4.76	6.85	9.7	22.7
	Inter	8.95	6.46	7.21		
Serine	Intra	5.1	4.7	6.51	12.8	42.8
	Inter	6.24	4.36	9.73		
Glutamine	Intra	5.04	5.67	4.62	12.1	22
	Inter	15.2	5.1	10.35		
Arginine	Intra	10.79	6.06	6.65	19.3	34.1
	Inter	10.56	8.04	6.04		
Tyrosine	Intra	2.93	4.38	5.32	10.5	61
	Inter	5.62	3.21	10.22		
Alanine	Intra	9.16	5.64	6.98	14.7	55.8
	Inter	9.21	4.51	11.29		
Tryptophan	Intra	3.34	5.07	3.42	22.7	152.6
	Inter	4.04	3.35	6.16		
Methionine	Intra	3.58	4.42	4.74	14.7	43.4
	Inter	6.89	3.5	9.71		
Valine	Intra	1.52	3.66	4.98	17.4	25.4
	Inter	5.04	5.19	9.48		
Phenylalanine	Intra	4.57	3.81	4.96	9.5	40.6
	Inter	6.57	3.89	7.04		
Isoleucine	Intra	3.51	3.59	7.89	15.5	45.5
	Inter	5.17	4.76	6.41		
Leucine	Intra	2.14	2.89	4.56	14.4	44
	Inter	5.87	3.23	7.98		

<sup>a</sup>CV: coefficient of variation; <sup>b</sup>Intra: intra-assay (n = 3); <sup>c</sup>Inter: inter-assay (n = 3 days; n = 3 replicates); <sup>d</sup>CVi: coefficient of variation within-subject biological variation<sup>(21)</sup>;

<sup>e</sup>CVg: coefficient of variation between-subject biological variation<sup>(21)</sup>.

TABLE 5 – Recovery

Compound	*Recovery %		
	Concentration 1	Concentration 2	Concentration 3
Aspartate	94.3 ± 9.8	104.8 ± 5.1	99 ± 10.3
Glutamate	103.7 ± 7.9	98.8 ± 3.5	108.6 ± 2.1
Asparagine	107.8 ± 5.9	87.9 ± 5.6	105.2 ± 5.5
Histidine	91.1 ± 8.4	104.4 ± 2.3	109.7 ± 6.3
Serine	105.1 ± 6.8	90.2 ± 3.3	97.7 ± 9.4
Glutamine	101.7 ± 6.9	104.6 ± 4.3	104.9 ± 6.3
Arginine	106.3 ± 5.4	112.3 ± 6.1	103.2 ± 5.8
Tyrosine	99.8 ± 6.1	95.8 ± 1.5	100.8 ± 10.9
Alanine	101 ± 8.8	106.9 ± 2.3	96.3 ± 8.6
Tryptophan	85.8 ± 3.4	93.1 ± 0.8	97.1 ± 6.3
Methionine	104.7 ± 7.9	99.1 ± 1.7	95.6 ± 9.8
Valine	104.6 ± 5.9	105.3 ± 5.4	106.7 ± 7.5
Phenylalanine	103.8 ± 7.2	98.9 ± 3.2	102.6 ± 7.2
Isoleucine	100.8 ± 5.6	97.6 ± 4.8	96.6 ± 4.9
Leucine	106.8 ± 7	98.4 ± 3.1	106.1 ± 8.8

\*n = 3.

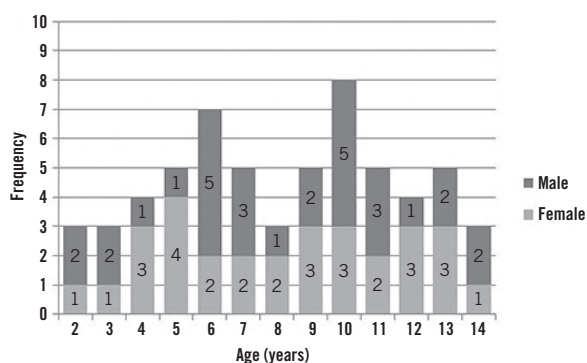


FIGURE 2 – Distribution of individuals according to their gender and age groups. The heights of the bars represent the frequencies of children for each gender

TABLE 6 – Reference ranges for plasma amino acids

Amino acids (nmol/ml)	Reference intervals	
	Lower limit (CI 90%)	Upper limit (CI 90%)
Aspartate <sup>a</sup>	3 (3-4)	20 (16-24)
Glutamate <sup>a</sup>	55 (47-65)	341 (294-397)
Asparagine	17 (12-24)	95 (87-102)
Histidine	38 (26-50)	158 (144-170)
Serine	56 (47-65)	166 (155-175)
Glutamine <sup>b</sup>	367	1252 (1103-1363)
Arginine	35 (28-43)	129 (120-137)
Tyrosine	32 (25-38)	106 (97-114)
Alanine <sup>a</sup>	167 (152-186)	604 (533-669)
Tryptophan <sup>a</sup>	34 (30-38)	102 (90-115)
Methionine	13 (11-15)	31 (29-33)
Valine	125 (112-140)	280 (265-295)
Phenylalanine	36 (32-40)	78 (73-82)
Isoleucine	35 (32-39)	79 (75-83)
Leucine <sup>b</sup>	70 (62-78)	160 (150-168)

CI: confidence interval; <sup>a</sup>: interval by robust method calculated after logarithmic transformation; <sup>b</sup>: interval calculated by Horn and Pesce method (2005)<sup>(17)</sup>.

Using the method of Reed to identify outliers, a unique value for histidine was considered suspect. The Clinical and Laboratory Standards Institute (CLSI)<sup>(16)</sup> recommends that, unless it is a clinically aberrant observation, the value should be kept in the sample and, therefore, this data was not excluded in the analysis.

The data obtained was compared with the control group values published by Strauss *et al.* (2010)<sup>(12)</sup>. In this work the authors utilized a group of 51 healthy Mennonites children to establish reference intervals and to monitor children with maple syrup urine disease, an inborn error of metabolism disorder of branched-chain amino acids. The study was chosen for comparison because it used similar analytical technique and sample size. Nevertheless, statistically significant differences in seven out nine amino acids analyzed were found (Table 7). These findings can be explained by differences in population and exclusion criteria, since the authors only excluded children with organic acidemia, and other metabolic diseases were probably included. However it is more likely that the statistical differences are related to analytical method. According to Shih (2003)<sup>(13)</sup>, serum amino acids can demonstrate important variations between laboratories, even when similar analysis tools are used and, therefore, the establishment of own reference intervals by each laboratory is indicated. This procedure results in tests with greater reliability and eliminated biases that might interfere with the analysis of the results of clinical research. If such study is not possible, it is recommended the use of revalidation techniques of reference ranges from other laboratories, but with greater caution in interpreting the results<sup>(16)</sup>.

The comparison with literature data was limited by the fact that in the study of Strauss *et al.* (2010)<sup>(12)</sup> the amino acids aspartate, glutamate, asparagine, serine, arginine and alanine were not analyzed. The study of Gregory *et al.* (1986)<sup>(9)</sup> was not utilized for statistical analysis because it does not present similar analytical

TABLE 7 – Comparison of amino acids values with international literature data

Amino acids	Measures of Central tendency (DP/min-max)		p value
	Present study	Strauss <i>et al.</i> (2010) <sup>(18)</sup>	
Histidine	100 (29)	87 (29) <sup>a</sup>	0.001
Glutamine	627 (335-1415)	527 (146) <sup>b</sup>	0.000
Tyrosine	72 (18)	63 (24) <sup>a</sup>	0.000
Tryptophan	61 (17)	50 (19) <sup>a</sup>	0.000
Methionine	22 (5)	31 (10) <sup>a</sup>	0.000
Valine	204 (38)	207 (65) <sup>a</sup>	0.517
Phenylalanine	58 (10)	55 (18) <sup>a</sup>	0.027
Isoleucine	57 (11)	65 (25) <sup>a</sup>	0.000
Leucine	113 (80-177)	119 (38) <sup>b</sup>	0.204

DP: standard deviation; <sup>a</sup>: Student's t test; <sup>b</sup>: Wilcoxon test.

methodology. The same occurred with the study of Lepage *et al.* (1997)<sup>(10)</sup> due to lack of descriptive data, such as normality test, mean, median, minimum and maximum, necessary to statistical analysis. No appropriate study describing reference intervals for amino acids in children was found in national literature to comparison.

intervals should be use in the Brazilian population in substitution to the international reference values, in order to eliminate the population bias. Once the early diagnosis is very important for better outcomes, reference intervals for plasma amino acids in Brazilian children aged between 0 and 2 years should also be established.

## CONCLUSION

The validated method demonstrated satisfactory linearity, precision, recovery, limits of quantification and detection, and it can be applied in the routine clinical practice. The study suggests that each laboratory should adopt its own reference intervals for amino acids in plasma. Even so, if this is not possible, the described reference

## ACKNOWLEDGMENTS

The authors thank to the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) for the financial support and master degree scholarship and to Hermes Pardini laboratory for the partnership in method development and amino acids analysis of the children included in this study.

## RESUMO

**Introdução:** A cromatografia líquida de alta eficiência é uma técnica utilizada para quantificação de aminoácidos no plasma. A definição de intervalos de referência na população é importante para o diagnóstico e o monitoramento de indivíduos com aminoacidopatias. **Objetivos:** Os objetivos do presente estudo foram validar um método de quantificação de aminoácidos e definir intervalos de referência em crianças brasileiras. **Resultados:** Boa separação cromatográfica foi obtida utilizando uma coluna de núcleo sólido C18. O método apresentou linearidade, limites de detecção e quantificação, precisão e acurácia satisfatórios. Os intervalos de referência para aspartato, glutamato, asparagina, histidina, serina, glutamina, arginina, tirosina, alanina, triptofano, metionina, valina, fenilalanina, isoleucina e leucina foram definidos em um grupo de 60 indivíduos saudáveis com idade entre 2 e 14 anos. **Conclusão:** A técnica apresentada pode ser aplicada na prática clínica. Cada laboratório deve, preferencialmente, estabelecer os próprios intervalos de referência. Se não for possível, recomenda-se a utilização dos intervalos de referência descritos neste estudo para o diagnóstico e o monitoramento de crianças brasileiras nessa faixa etária.

**Unitermos:** cromatografia líquida; erros inatos do metabolismo; aminoácidos; valores de referência.

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