RESEARCH ARTICLE

Establishment of Reference Value of 20 Amino Acids for Toddlers by High Performance Liquid Chromatography Tandem Mass Spectrometry

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Received date: Jan 22, 2024; Revised date: Apr 1, 2024; Accepted date: Apr 5, 2024

Abstract

BACKGROUND: Amino acids are one of the essential metabolites, especially the 20 amino acids that are preserved as the building blocks of protein. Alterations in amino acid concentrations are related to disease such as inborn error of metabolism, cancer, as well as nutritional status. Hence, it is necessary to define reference values of 20 plasma-free amino acids for Indonesian toddlers and to establish a robust measurement technique using chromatography with tandem mass spectrometry (MS).

METHODS: This study was a cross-sectional preliminary study to establish reference values. The sample was prepared by mixing plasma with 20% sulfosalicylic acid. Plasma-free amino acids were measured with high-performance liquid chromatography (HPLC) non-derivatization technique using column XTerra for chromatographic separation coupled with tandem MS. Amino acids reference values were taken from 101 healthy Indonesian toddlers aged 1-3 years old. Since amino acids data were not Gaussian distributed, the lower and upper of the reference value was established from the 5th percentile and the 95th percentile, respectively.

RESULTS: Analysis for 20 amino acids was validated. The accuracy ranged from 90.53-105.39% and the precision ranged from 0.06-3.80%. The limit of detection range was 1-2 nmol/mL, and the limit of quantification range was 2-4 nmol/mL. The result was linear, with R^2 higher than 0.998. There was no significant difference between boys and girls for all amino acids except for glycine.

CONCLUSION: HPLC with tandem MS method can be used to evaluate amino acids in clinical practice. The reference values obtained are specific for aged 1-3 years old from urban areas in Indonesia. The study suggests that for each population, the reference values for amino acids should be established.

KEYWORDS: amino acids, high-performance liquid chromatography, tandem mass spectrometry, reference values, Indonesia

Indones Biomed J. 2024; 16(2): 172-9

Introduction

Amino acids are one of the essential metabolites in the human body. Twenty amino acids are the building blocks of protein and serve as the key precursors for synthesis of hormones, neurotransmitters, muscle growth, and other cellular processes.(1) Alterations in amino acid concentrations are related to several inborn errors of metabolism (IME), such as phenylketonuria (PKU) and maple syrup urine disease

(MSUD), cancer patient, and some nutritional conditions like stunted growth.(2-5) Low nutrient intake was found among toddlers which caused micronutrients deficiency. (6) Amino acid metabolism disorder can cause severe morbidity and mortality. Amino acid analysis becomes crucial because delayed diagnosis and treatment can result in severe consequences, especially in children.(7,8) Amino acid analysis is also crucial in the evaluation of treatment of the disease, which in some cases is required to be done routinely since childhood.(9)

Amino acid analysis commonly uses physiological specimens such as plasma, urine, and cerebrospinal fluid.(9) The commonly used methods are ion-exchange chromatography (IEC) accompanied by post-column derivatization or pre-column derivatization before reversed-phase high-performance liquid chromatography (RP-HPLC). Although the IEC method has been the gold standard, this method has some disadvantages such as long runtime, burdensome, expensive, and hard to perform in a clinical laboratory. The HPLC has become the alternative because it is relatively inexpensive, simple, requires shorter analysis time, and can be done with or without derivatization.(10-12)

The reference values are needed to determine the interpretation of amino acid levels in each subject. However, the reference values of amino acids for children aged 1-3 years old in Indonesia have not yet been established. The interpretation commonly relies on the reference values from international literature. Since amino acid levels are influenced by various factors, such as age, genetics, race, nutrition, medications, and toxins, relying on the reference values derived from other populations may mislead the interpretations.(8) Nowadays, the need for the concept of individual-based precision nutrition has arisen. (13) Therefore, this study was conducted to validate the measurement technique of plasma-free amino acids using the HPLC with tandem mass spectrometry (MS) and a preliminary study of reference values of twenty plasma-free amino acids for Indonesian toddlers aged 1-3 years old.

Methods

Study Design and Subjects Recruitment

This was a cross-sectional study conducted in Cipto Mangunkusumo Hospital and Jakarta Provincial Public Health Laboratory DKI Jakarta, Indonesia, from August 2016 to March 2017. This was a preliminary study to establish a reference value of 20 amino acids from 101 healthy Indonesian children aged 1-3 years old (50 girls and 51 boys) who lived in an urban area in Jakarta. All subjects included had no fever, cough, runny nose, or diarrhea and had normal findings on physical examination by pediatricians. All subjects were not stunted (Z score >-2SD on WHO height/length-for-age curve) or severely wasted (Z score >-3SD on WHO weight-for-height and weight-for-age curve) confirmed by trained research staff. There were no subjects diagnosed with chronic diseases, such as tuberculosis and chronic kidney disease, in the treatment of certain diseases, or had a particular genetic and metabolic disease from history. All subjects were born full-term, appropriate for gestational age, and had normal birth weight. There were no subjects with ascariasis or other stool parasites confirmed with fecal analysis and had C-reactive protein (CRP) levels under 20 mg/dL. Food dairy of 3x24 hours was documented into case records forms for each subject and was confirmed by a dietician. The protocol of this study was approved by the Ethics Committee Faculty of Medicine Universitas Indonesia (No. 265/UN2.F1/ETIK/2016), and informed consent was obtained from the parents.

Preparation of Plasma Samples

Blood sample was drawn by venous puncture and placed in a 3 mL container tube with K3EDTA anticoagulant. The blood sample was collected after the subject had been fasting for 10 hours. The parents were instructed to halt feeding the children from 9 PM to 7 AM, as 7 AM was the time when the subjects were scheduled to have their blood samples taken. The blood samples were centrifuged at 2,010 x g for 15 minutes, and the plasma was separated and stored at -80^{o}C until the analysis day. The plasma was thawed at room temperature when ready to be analyzed. Afterward, 250 µL of 20% sulfosalicylic acid (PT Segara Husada Mandiri, Jakarta, Indonesia) was added to the 250 µL plasma sample for the deproteinization and then homogenized on a vortex for 1 minute. The sample was then centrifuged at 5,000 g for 15 minutes at 4^{o}C. The supernatant was collected into a vial and ready for HPLC-MS/MS analysis.

Analysis of Plasma Free Amino Acids

Analysis of amino acids were performed on Alliance e2695 Separation Module HPLC system (Waters Corporation, Milford, MA, USA) using the non-derivatization technique with column XTerra MS C18 with 3x150 mm ID and 3.5 µm particle size (Waters Corporation) combine with Tandem Mass Quatro Micro Spectrometer (Waters Corporation) using the selected ion recording (SIR) method. The data were acquired using MassLynx Mass Spectrometry

Software (Waters Corporation). The column was set at the temperature of 40°C. The sample was injected in a volume of 30 µL. The amino acids were separated at the flow rate of 0.15 mL/minutes using a gradient with solvent A (0.1% heptafluorobutyric acid in water 1:9) and solvent B (acetonitrile (Merck, Billerica, MA, USA) mixed with 0.1% heptafluorobutyric acid). At initial, solvent A was set at 90%, and solvent B was set at 10%. After 3.7 minutes, solvent A was set at 55% and solvent B at 45%. From minute 4.00 to 4.9, solvent A was set at 5% and solvent B at 95%. From minute 4.9 forward, Solvent A was set at 90%, and solvent B was set at 10%.

The standard solution consisted of 19 amino acid standards and a separate standard solution for asparagine. The 19 amino acids standard solutions consist of L-alanine, B-alanine, L-arginine, L-aspartic acid, L-cysteine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, L-hydroxyproline, and L-lysine. All the standard solutions were stored at 2-8°C. The standard solution were diluted in 0.1 N HCl and prepared to undergo gradual measurement, starting from 0.5 nmol/mL until the equipment could detect the amino acids.

Preparation for a single amino acid standards L-asparagine which obtained from Sigma Aldrich (St. Louis, MO, USA) were prepared by dissolving the standard solutions in 0.1 N HCl (Merck) until the concentration of 2500 nmol/mL as standard stock solution. The stock solutions were diluted to undergo gradual measurement, starting from 1 nmol/mL until the equipment could detect the asparagine.

To determine isoleucine and leucine, single standard isoleucine and leucine from European Directorate for Quality of Medicines and HealthCare (Strasbourg, France) was used. To determine retention time between L-alanine and B-alanine, single standard L-alanine (Sigma Aldrich) and heptafluorobutyric acid (Sigma Aldrich) were used.

Method Validation of Plasma Free Amino Acids

Validation method was established before performing the samples according to Food and Drug Administration (14) such as the linearity, the limit of detection (LOD) and the limit of quantification (LOQ), accuracy, and precision were performed. The lowest concentration detected by the equipment was 2 nmol/mL for 19 amino acids and 10 nmol/mL for asparagine, then it was performed for 10 times to obtain the mean and standard deviation. The LOD and LOQ were determined by the mean concentration and standard deviation of each amino acid in the lowest concentration detected by the equipment. The LOD was calculated by the mean concentration plus three times the standard deviation. The LOQ was calculated by the mean concentration plus ten times the standard deviation. The linearity for 19 amino acids were evaluated by water as blank and following eight concentration amino acid standard (0, 20, 40, 60, 80, 120, 140, 160 and 180 nmol/mL). The linearity of asparagine was evaluated by water as blank and following nine concentrations for asparagine (0, 10, 50, 100, 300, 500, 700, 900, 1000, 1500) nmol/mL. The peak area of each amino acid was plotted to form the standard curve.

The accuracy and repeatability for each amino acids were determined by using three levels of the standards solution for 19 amino acids (40 nmol/mL, 80 nmol/mL, 120 nmol/mL) and four levels of the standards solution for asparagine (10, 200, 400, 600 nmol/mL). Each standard solution was analyzed ten times. The accuracy was defined as the agreement between the obtained value and the actual concentration of each amino acid. The acceptable criteria should be within 15% of nominal concentration. The repeatability was determined by calculating the coefficient of variation (CV). The acceptable criteria should not exceed 15%. Carry-over was determined by measuring the peak area of the blank water after the analysis. Then the reproducibility analysis was performed by analyzing standard solution on three consecutive days.

Statistical Analysis

Data analysis was performed using R Studio version 1.1.442 (Posit, Boston, MA, USA) which the open system of statistic software downloaded from *https://cran.r-project.org/*. The Gaussian distribution was assessed using the Shapiro-Wilk test, and the result showed that the data had non-normal distribution. Gender differences were determined using the Mann-Whitney U test. The references limits were determined as value±standar deviation (SD). Since all the amino acids data were not Gaussian distributed, the value of each amino acids was ranked by percentiles, then the lower and upper limit of the reference value were established from the 5th percentiles and 95th percentiles, respectively.

Results

Method Validation

The total run time for the analysis was 15 minutes. Each individual chromatogram of the standard solution showed satisfactory peaks, indicated by the well resolved peak

shape (Supplementary 1). Analysis of one of the samples was also well resolved, with each individual chromatogram of one of the samples showed a satisfactory peak shape (Supplementary 2).

The linearity of the calibration curve of each individual amino acid was shown in Table 1. The regression coefficient was over than 0.998 in each amino acid range. The calibration curves showed good linearity. The LOD range was 1-2 nmol/mL, and the LOQ range was 2-4 nmol/mL for 19 amino acids and 10-20 nmol/mL, indicating that the method was sensitive.

Table 2 summarized the accuracy and precision of 19 amino acids, meanwhile Table 3 showed the accuracy of Asparagine. The accuracy ranged from 95.84% to 105.39%, 94.45% to 103.65%, and 98.72% to 100.84% in 40 nmol/mL, 80 nmol/mL, and 120 nmol/mL, respectively. This result showed that the accuracy of all individual amino acids was within the acceptable criteria. The precision has shown as the coefficient variation (CV) which ranged from 0.49% to 3.8%, 0.25% to 3.14%, and 0.07% to 2.06% and in 40 nmol/mL, 80 nmol/mL, and 120 nmol/mL, respectively. These results showed that the precision within acceptable criteria. All 20 amino acids were not detected from the water blank, indicating no related carry-over.

Reference Value

The results of 20 amino acids reference values for toddlers obtained from current study showed that there were no significant differences between girls and boys, except for glycine, which was significantly higher in girls (Table 4). Meanwhile, as comparison we also summarized amino acid reference values from other studies and laboratory (Table 5) Almost all amino acids in this study had lower reference values than other populations, except for aspartate, isoleucine, hydroxyproline, and asparagine.

Discussion

Our method had shown to allow the analysis of underivatized amino acid using HPLC system coupled with tandem mass spectrometry. This method could address the problem of moisture sensitivity in the derivatization process and the instability of the derivatives faced by other amino acid analysis methods.(15,16) The separation of underivatized amino acids in liquid chromatography (LC) usually can only be achieved using ion-pairing agents, such as trifluoroacetic (TFA) and nonafluoropentanoic acid (NFPA).(17) In our study, the amino acids were separated by underivatized amino acid using HFBA as the ion-pairing agent. Another non-derivatization technique, the hydrophilic interaction liquid chromatography (HILIC) has emerged as the alternative for HPLC. The HILIC method can avoid the problem of reduced ionization efficacy by suppression and contamination faced in LC. However, our method had the advantage of overcoming the inability

Table 1. Linearity, LOD, and LOQ of amino acids.

Amino Acid	Linearity R^2	Range	LOD (nmol/mL)	LOQ (nmol/mL)
Tryptophan	0.998	2 – 180	2	2
Arginine	0.999	2 – 180	2	2
Aspartate	0.999	2 – 180	2	3
Cysteine	0.999	2 – 180	2	2
Glutamate	0.998	2 – 180	2	3
Glycine	0.999	2 – 180	2	2
Histidine	0.999	2 – 180	2	3
Isoleucine	0.999	2 – 180	2	2
Leucine	0.999	2 – 180	2	2
Lysine	0.999	2 – 180	2	2
Methionine	0.999	2 – 180	2	3
Phenylalanine	0.999	2 – 180	2	2
Proline	0.999	2 – 180	2	3
Serine	0.999	2 – 180	2	2
Threonine	0.999	2 – 180	2	3
Tyrosine	0.999	2 – 180	2	4
Valine	0.999	2 – 180	2	2
Hydroxyproline	0.999	2 – 180	2	4
Alanine	0.999	2 – 180	2	4
Asparagine	0.999	10 – 1500	10	20

Table 2. Precision and accuracy of 19 amino acids.

Amino Acid	40 nmol/mL		80 nmol/mL		120 nmol/mL	
	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
Tryptophan	1.02	98.97	0.25	98.86	0.19	100.33
Arginine	2.59	101.66	2.12	101.35	1.82	99.68
Aspartate	1.76	105.39	0.53	98.67	2.06	100.84
Cysteine	2.14	95.84	1.95	99.64	1.70	100.02
Glutamate	3.80	103.01	1.39	103.65	2.21	100.37
Glycine	1.45	98.88	1.12	100.95	2.70	100.42
Histidine	2.84	99.97	0.92	99.62	0.97	99.80
Isoleucine	0.49	99.14	0.19	94.45	0.06	100.44
Leucine	0.18	98.90	0.39	99.16	0.41	99.36
Lysine	1.87	98.87	0.86	98.87	0.65	99.46
Methionine	2.60	100.94	2.25	99.69	1.27	100.26
Phenylalanine	0.66	100.24	0.35	99.05	0.33	99.23
Proline	2.64	99.07	1.96	99.14	0.07	100.57
Serine	2.86	100.58	1.56	100.59	0.91	99.96
Threonine	2.84	101.12	1.18	99.25	0.87	100.66
Tyrosine	1.74	100.02	1.45	100.12	0.41	100.36
Valine	1.93	102.44	1.09	100.97	0.76	99.77
Hydroxyproline	2.94	98.26	3.14	98.24	1.49	98.72
Alanine	3.50	101.96	1.73	99.80	1.16	99.05

to separate isoleucine/leucine pairs faced in HILIC as described. (17,18)

The method was able to demonstrate high accuracy with a short analysis time. This result is in concordance with the result from other studies using reversed LC-MS/MS and HILIC-MS/MS.(17-19) One study obtained 83-105% accuracy with total runtime of 20 minutes, and another one obtained an accuracy of 78-101% with total runtime of 18 minutes.(17,18) Compared to other methods, HPLC-MS/MS had several advantages and disadvantages. The method showed higher accuracy and twice shorter runtime than HILIC-MS as described.(17) However, HILIC-MS had its benefits in combining amino acid analysis with a more extensive set of other metabolites. Compared to gas chromatography (GC)-MS, HPLC-MS/MS showed somewhat similar accuracy but required longer analysis time.(16) However, GC-MS could not analyze arginine due to the thermal instability of the derivative. Our method also showed similar accuracy compared to RP-HPLC with fluorescence detection or with ultraviolet-visible (UV-Vis) detection as described by other studies.(12,16)

The results also met the acceptability criteria of precision, sensitivity, and linearity. The Food and Drug Administration established the criteria of precision to be less than 15%.(14) Our method showed similar precision arise compared to HILIC-MS/MS (0.8 to 6.9 %) but better compared to RLC-MS/MS and GC-MS methods (less than 13% and 12%, respectively).(17,18) The differences in precision might arise from retention time and MS signal drifting with time. The method also fulfilled the analytical sensitivity and linearity criteria as the LOD and LOQ were low enough to diagnose or evaluate amino acid metabolism disorders, and R^2 was close to 1. Two studies yielded similar LOD and linearity R^2 to this study as they used the same LC-MS/MS method despite different chromatography methods. (17,18)

Based on the performance characteristics, it can be concluded that the method is valid and reliable to produce an accurate result. As it is also relatively simple, the method can be used to evaluate amino acid disorders in clinical settings. Our method has some advantages and disadvantages compared to other analytical methods as mentioned above. Therefore, the choice of analytical

Table 3. Precision and accuracy of asparagine.

Amino Acid	10 nmol/mL		200 nmol/mL		400 nmol/mL		600 nmol/mL	
	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
Asparagine	3.64	90.53	3.29*	99.20	1.42	99.18	0.79	99.77

Table 4. Plasma amino acids reference values.

Amino Acid	Reference Values (nmol/mL)
Tryptophan	8.2–78.0
Arginine	10.0–78.5
Aspartate	7.1–71.6
Cysteine	2.0–12.0
Glutamate	11.2–149.4
Glycine (boys)	7.2–66.8
Glycine (girls)	10.5–78.1
Histidine	5.0–30.9
Isoleucine	8.0–144.0
Leucine	19.0–88.0
Lysine	6.2–105.7
Methionine	5.0–46.8
Phenylalanine	32.2–173.7
Proline	3.6–182.0
Serine	5.0–65.9
Threonine	5.1–124.6
Tyrosine	<97.5
Valine	23.5–199.1
Hydroxyproline	17.2–174.9
Alanine	49.0–237.3
Asparagine	60.4–304

methods may vary depending on the analysis target and the availability of the equipment and reagents.

The included subjects were expected to represent the population of Indonesian children aged 1-3 years old. The children included in this study were all healthy and free from factors associated with alteration in amino acid levels. The CRP test had excluded chronic diseases and inflammation. The fecal examination had excluded gastrointestinal parasitic investment disease. Number of male and female were also balanced. Based on inclusions and exclusion criteria and the number of included subjects, the result of our study can be used as provisional reference value.

As in other studies, plasma was used as the sample to describe the condition in the circulation. Dried blood spot is commonly used for metabolic disorders screening in children for its simplicity and cost-effectivity, while plasma is used for confirmation. Although still limited, recent studies showed that dried blood spots had a good overall agreement with plasma for amino acid analysis. Further studies are needed to confirm this finding, and if the agreements are good enough, the sample can then be drawn from a dried blood spot.(20-22)

This study found that there were no significant differences between males and females except for glycine. Sex differences affect protein metabolism due to changes in hormones and differences in body composition. Since prepubertal children have relatively similar hormone profiles and muscle mass, sex differences do not show a significant effect on their protein metabolism.(22)

This study found that almost all our amino acid reference values were lower than other populations (Table

Table 5. Plasma amino acids reference values from other studies.

Amino Acid	Lepage, *et al*[23] (µmol/L)*	Cruz, *et al*[24] (µmol/L)*	Yi, *et al*[12] (µmol/L)*	Mayo Clinic[25] (nmol/mL)
Tryptophan	35–73	34–102	2 1.7–78.8	23–80
Arginine	46–90	35–129	2 0.5–71.4	31–132
Aspartate	3–8	3–20	1.1–5.9	<11
Cysteine	NA	NA	NA	2–36
Glutamate	25–81	55–341	21.9–80.2	22–131
Glycine	138–276	NA	114.2–304.2	149–417
Histidine	61–91	38–158	40.9–88.7	12–132
Isoleucine	4–78	35–79	36.9–121.8	30–111
Leucine	79–147	70–160	3.6–213.4	51–196
Lysine	88–172	NA	51.3–176.5	59–240
Methionine	13–22	13–31	4.6–38.6	11–37
Phenylalanine	39–65	36–78	38.3–84.0	30–95
Proline	93–220	NA	59.8–277.0	80–357
Serine	97–154	56–166	65.0–165.3	71–208
Threonine	61–115	NA	39.1–199.8	58–195
Tyrosine	40–77	32–106	32.2–124.4	31–106
Valine	147–255	125–280	145.2–365.9	106–320
Hydroxyproline	NA	NA	4.3–116.7	7–35
Alanine	173–349	167–604	203.4–521.2	144–557
Asparagine	29–56	17–95	17.3–46.7	29–87

*µmol/L=nmol/mL. NA: Not available.

5). Unlike most of other studies, this study used tandem mass spectrometry instead of spectrophotometry. This difference in detection methods might cause differences in the results. As most amino acid concentrations increase throughout childhood, the differences could also arise due to differences in the age range. The age range in those studies was two years old, 2 to 14 years old, and 1 to 5 years old.(12,23-25).

The lower amino acid reference values in this study might also be related to low energy intake. One study showed that the total energy and protein intake of Indonesian children aged 1-3 years old was 910 kcal/day and 30g/day.(26) This total energy intake did not meet the recommended daily intake for Indonesian children aged 1-3 years old, 1350 kcal/day.(27) Although the total protein intake exceeded the recommended daily intake (20 g/day), the amino acid values remained low because the protein was used to fulfill the total energy requirement. The limitation of this study includes the difficulty of implementing fasting, especially for children who are still consuming breast milk.

To authors' knowledge, this is first study to analyze amino acid reference values in toddler in Indonesia. This result is specific for toddlers living in Jakarta urban areas. Since amino acid levels are affected by various nutritional and environmental factors, the values may vary from different populations. Therefore, we suggest that each population should establish its reference values.

Conclusion

A reversed phase-HPLC with tandem MS method had been validated and could be used to evaluate amino acids in clinical practice. The amino acids had no difference level between boys and girls, except for glycine. The reference values obtained are specific for aged 1-3 years old from urban areas in Indonesia, which had been demonstrated to be lower compared to other studies and laboratories that might be affected by various nutritional and environmental factors.

Acknowledgments

The authors thank to Ms. Rina Rahayuningsih (Jakarta Provincial Public Health Laboratory DKI Jakarta, Indonesia) for her assistance.

Authors Contribution

MMP was involved in the conceptualization, data curation, formal analysis, funding acquisition, project administration, visualization, writing-original draft, writing-review and editing. SI, DRS, IST, SGM, and MM were involved in the conceptualization, formal analysis, funding acquisition, visualization, writing-review and editing. ES was involved in data curation, formal analysis, and visualization. All authors have agreed with the final revision of the manuscript.

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