Liquid chromatography

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Submission date: 26-Oct-2022 08:47AM (UTC+0700)
Submission ID: 1935507337
File name: Liquid_chromatography.pdf (634.31K)
Word count: 3827
Character count: 19568

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Liquid chromatography-tandem mass spectrometric analyses of the amino acid profiles of normal and malnourished children treated at Cipto Mangunkusumo Hospital

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Abstract Children require dietary protein for growth. The nutritional quality of proteins is influenced by their amino acid composition. Amino acids are required for life, contributing to homeostasis, immunity, growth, and development. In this preliminary study, we used liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine the amino acid profiles of glycine, alanine, proline, valine, leucine, ornithine, methionine, phenylalanine, arginine, citrulline, tyrosine, aspartic acid, and glutamic acid of normal and malnourished children treated at Cipto Mangunkusumo Hospital. A total of 60 subjects were analyzed from December 2016 to April 2017. Dry blood spots were analyzed using LC-MS/MS. The study subjects comprised 12 malnourished and 18 normal children. The mean weight, height, and z-score (weight-for-age, height-for-age, and weight-for-height) were significantly lower in the malnourished group. The coefficients of variation percentage among runs ranged from 1.76% to 12.03%. There was no significant difference between the amino acid profiles of the normal and malnourished groups, although the concentrations of glycine and glutamate were significantly higher in the malnourished group.

1. Introduction

Malnutrition, a serious health problem affecting children both in developed and developing countries [1], contributes to 300,000 annual deaths and to 50% of child mortality worldwide [2-5]. The World Health Organization (WHO) reported that 35.9 million children in Asia, younger than 5 years, exhibit wasting and that 12.6 million children show symptoms of severe wasting [6]. In 2013, the Basic Health Research Agency of Indonesia (Riskesdas) reported that there was an increasing prevalence of wasting and severe wasting (17.9% and 19.6%, respectively) in children younger than 5 years in 2010 [7].

2. Materials and Methods

This descriptive and analytical study employed a cross-sectional design. The study protocol had been approved by the Health Research Ethics Committee, Faculty of Medicine Universitas Indonesia-Cipto Mangunkusumo Hospital. Study participants included 1- to 3-year-old children residing in the

Community Health Center of Jatinegara region and admitted to the Kiara Polyclinic at the Cipto Mangunkusumo Hospital from December 2016 to January 2017. At study inclusion, each patient's body weight, height, and length were measured. The nutritional status (z-score) was calculated from these variables using the WHO AnthroPlus software. Nutritional status was considered normal if the z-scores ranged from less than –2 standard deviations (SD) to less than 1 SD, according to the calculations of weight-for-age (WA), height-for-age (HA), and weight-for-height (WH). Malnutrition was affirmed if the z-score was greater than –2 SD, calculated according to WA, HA, WH (moderate malnutrition). Children with a diagnosis of an acute infection causing fever or loss of appetite or conditions that adversely affect nutrition, such as diarrhea, respiratory tract infection, measles, malaria, dengue fever, and abnormalities of carbohydrate, amino acid, or lipid metabolism, were excluded from the study. In addition, children with other congenital diseases or syndrome were excluded from the study.

Informed consent was obtained from parents of the included children. Venous blood was drawn from each patient, and 100 µL of K_3EDTA was dripped on a filter paper using a calibrated fixed-volume pipette to obtain a dry blood spot (DBS) sample. The pipette tip was held a few millimeters from the paper, and blood was dripped into a circle drawn on the paper (Whatman 903, GMS). The filter paper had two circles, each with a diameter of 1.5 cm. Filter papers with dripped blood were stored at room temperature (15 ºC–30 °C) for 24–48 h. For longer storage, the samples were kept at less than –18°C and <30% humidity. It is recommended that the sample with dripped blood should be inserted into a vacuum bag with a desiccant and a humidity indicator [8].

Amino acid analysis was conducted by injecting the samples directly into the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system (Xevo TQD Tandem, Triple Quadrupole Mass Spectrometer; Waters Corporation, Milford, MA, USA), without separating the sample components using the LC system. Separation was performed using an extraction process. Analyte concentrations were measured and then calculated using an internal standard (IS). Quality control was performed using two different concentrations of the IS. The following amino acids were measured (µmol/L): glycine, alanine, proline, valine, leucine, ornithine, methionine, phenylalanine, arginine, citrulline, tyrosine, aspartic acid, and glutamic acid [9].

3. Results

The within-run precision of amino acid concentration measurements were determined from five consecutive tests performed on the same day (Table 1).

Table 1. Within-run tests of the precision of measuring amino acid concentrations.

No	*Within run*												
	Gly	Ala	Pro	Val	Leu	Orn	Met	Phe	Arg	Cit	Tyr	Asp	Glu
	µmol/ Liter	µmol/ Liter	µmol/ Liter	µmol/ Liter	µmol/ Liter	µmol/ Liter	µmol/ Liter	µmol/ Liter	µmol/ Liter	µmol/ Liter	µmol/ Liter	µmol/ Liter	µmol/ Liter
1	344.64	349.44	144.12	187.79	161.32	129.56	35.93	69.9	53.47	35.15	62.88	49.89	162.54
2	340.52	342.11	143.13	197.15	159.4	120.09	33.91	69.33	50.54	35.68	67.06	42.03	167.03
3	358.45	329.84	145.62	197.04	153.51	121.74	32.86	65.90	51.66	35.64	61.07	51.64	167.66
4	372.33	321.37	146.58	190.6	153.72	131.93	35.82	66.89	50.54	34.76	62.99	52.99	172.14
5	377.57	336.20	149.77	182.45	156.17	128.03	34.92	65.05	54.70	34.08	62.31	59.08	161.64
Mean	358.70	335.79	145.84	191.01	156.82	126.27	34.69	67.41	52.18	35.06	63.26	51.13	166.20
SD	16.36	10.84	2.57	6.28	3.46	5.12	1.31	2.12	1.85	0.67	2.26	6.15	4.25
CV(%)	4.56	3.23	1.76	3.29	2.21	4.05	3.77	3.15	3.54	1.90	3.57	12.03	2.56

The characteristics and test results of study participants are presented in Table 2.

Table 2. Characteristics of study participants (n = 57)

Characteristics	Malnourished children	Normal children	†p value
Sex			
Male	7 (41.2%)	10 (58.8%)	
Female	5 (38.5%)	8 (61.5%)	
Age (months)‖	22.83 (±6.38)	22.79 (±6.36)	0.963
Weight (kg)‖	8.09 (±0.97)	10.39 (±1.17)	0.008*
Height (cm)‖	77.30 (±4.90)	82.48 (±4.78)	0.000*
z-score (WH)†	−2.43 (±0.27)	−0.73 (−1.42 to 0.44)	0.000*
z-score (WA)	−3.16 (±0.37)	−0.98 (±0.65)	0.000*
z-score (HA)	−2.76 (±0.73)	−1.07 (±0.51)	0.000*

W, weight; H, height; A, age
‖Unpaired *t* test
†Mann–Whitney test
*P < 0.05

Amino acid profiles of malnourished and normal children

The amino acid profiles of malnourished and normal children were divided into amino acids that are essential (valine, leucine, methionine, phenylalanine, and arginine) (Table 3) and non-essential (glycine, alanine, proline, ornithine, citrulline, tyrosine, aspartic acid, and glutamic acid) (Table 4).

Table 3. Essential amino acid profiles of malnourished and normal children

Essential amino acid	Undernutrition children	Normal children	†p value
Valine† (µmol/L)	122.1 (91.8–130.7)	115.9 (83.9–284.6)	0.819
Leucine† (µmol/L)	111.5 (±24.5)	106.3 (75.2–355.4)	0.755
Methionine‖ (µmol/L)	17.0 (±2.1)	17.1 (±3.5)	0.927
Phenylalanine† (µmol/L)	46.0 (40.2–79.1)	45.7 (38.8–68.3)	0.602
Arginine‖ (µmol/L)	13.1 (±6.6)	15.8 (±7.1)	0.301

‖Unpaired *t* test
*p < 0.05
†Mann–Whitney test

Table 4. Profiles of non-essential amino acids of malnourished and normal children

Non-essential amino acid	Malnourished children	Normal children	†p value
Glycine (µmol/L)	187.1 (±67.9)	137.4 (±42.2)	0.019*
Alanine‖ (µmol/L)	196.3 (±38.5)	203.8 (±55.3)	0.686
Proline‖ (µmol/L)	138.3 (±48.0)	122.3 (±31.1)	0.274
Ornithine† (µmol/L)	118.2 (±43.1)	120.6 (82.9–217.5)	0.305
Citrulline‖ (µmol/L)	25.5 (±9.1)	28.0 (±8.5)	0.435
Tyrosine† (µmol/L)	39.2 (±8.1)	38.8 (28.0–68.8)	0.787
Aspartic acid† µmol/L)	61.0 (1.4–2364.0)	42.9 (1.2–1440.7)	0.285
Glutamic acid† (µmol/L)	242.5 (±42.1)	192.0 (151.1–369.4)	0.015*

‖Unpaired *t* test
*p < 0.05
†Mann–Whitney test

We compared our present data with reference values of children from Malaysia, Singapore, and the United States (Table 5).

Table 5. Amino acid profiles of malnourished and normal children compared with reference values of other populations

Amino acid	Malnourished children	Normal children	Malaysia *	Singapore †	Texas††
Valine (µmol/L)	122.1 (91.8–130.7)	115.9 (83.9–284.6)	70–348	50–375	71–329
Leucine (µmol/L)	111.5 (±24.5)	106.3 (75.2–355.4)	29–266	60–260	44–184
Methionine (µmol/L)	17.0 (±2.1)	17.1 (±3.5)	9–42	0–54	15–57
Phenylalanine (µmol/L)	46.0 (40.2–79.1)	45.7 (38.8–68.3)	18–180	20–130	39–126
Arginine (µmol/L)	13.1 (±6.6)	15.8 (±7.1)	0.53–21	0–160	1–32
Glycine (µmol/L)	187.1 (±67.9)	137.4 (±42.2)	76–725	120–480	184–649
Alanine (µmol/L)	196.3 (±38.5)	203.8 (±55.3)	67–917	150–650	136–516
Proline (µmol/L)	138.3 (±48.0)	122.3 (±31.1)	NA	66–333	108–282
Ornithine (µmol/L)	118.2 (±43.1)	120.6 (82.9–217.5)	NA	40–160	49–270
Citrulline (µmol/L)	25.5 (±9.1)	28.0 (±8.5)	3.3–35	5–54	7–32
Tyrosine (µmol/L)	39.2 (±8.1)	38.8 (28.0–68.8)	10–182	30–130	46–204
Aspartic acid (µmol/L)	61.0 (1.4–2364.0)	42.9 (1.2–1440.7)	NA	0–80	N/A
Glutamic acid (µmol/L)	242.5 (±42.1)	192.0 (151.1–369.4)	68–557	0–200	182–532

*Reference values from the Institute of Medical Research, Kuala Lumpur, Malaysia; Waters liquid chromatography-tandem mass spectrometry (LC-MS/MS) system; dropped blood sample (DBS).
†Reference values from the National University Hospital; LC-MS/MS method, plasma sample
††Reference value from Core Laboratory St Louis Children's Hospital, Texas; Waters LC-MS/MS method; DBS sample

Discussion

The present preliminary study aimed to determine the amino acid profiles of malnourished and normal children as a component of a larger study of plasma-free amino acid profiles, insulin-like growth factor-1 concentrations, and the gene polymorphisms of stunted children. Here we compared amino acid concentrations of malnourished with those of normal children.

The present study employed a DBS method. A capillary blood sample was not used to avoid acquiring multiple samples from the subjects. Bloom et al. found that amino acid concentrations determined using the DBS method significantly correlate, although they are negatively biased, compared with the plasma concentrations of amino acids determined using ultra-performance liquid chromatography [10]. Moreover, Bloom et al. found that DBS amino acid concentrations were stable for at least 3 days at temperatures up to 65ºC, enabling samples to be readily transported to the laboratory for analysis [10].

Within-run precision tests using the authors' blood samples were performed consecutively five times on the same day, immediately before the examination. We compared our CV data with those of Held, Ulrike, and Prinsen et al.[11-13] Our CVs were consistent with those acquired using quantitative LC-MS/MS analyses conducted by Held, although the within-run CVs were <10%. Aspartate had a lager CV (12.03%), which may be explained by the lower concentration of circulating aspartate versus other amino acids. This CV value was similar to that of ornithine determined by Ulrike [12], whereas the larger CV of aspartate determined by Prinsen et al. might be explained by the sample preparation technique, instrument calibration, instrument optimization, adverse environmental factors in the laboratory, and sample

homogeneity on filter papers. Comparisons of the CV determined here versus the studies cited above [11-13] are presented in Table 6.

Table 6. CV (%) comparisons to the studies of others

Amino acid	Present Study CV (%)	Held Study CV* (%)	Ulrike Study CV† (%)	Prinsen Study CV‡ (%)
Glycine	4.56	<10	3.8	3.8
Alanine	3.23	<10	3.7	1.9
Proline	1.76	<10	8.4	2.4
Valine	3.29	<10	4.3	6
Leucine	2.21	<10	4.0	2.4
Ornithine	4.05	<10	10.1	2.9
Methionine	3.77	<10	3.2	1.9
Phenylalanine	3.15	<10	3.7	2.3
Arginine	3.54	<10	6.2	1.7
Citrulline	1.90	<10	5.2	4
Tyrosine	3.57	<10	4.3	3.8
Aspartic acid	12.03	<10	2.8	6.9
Glutamic acid	2.56	<10	5.9	3.4

*LC-MS/MS method using an API 4000 Shimadzu HPLC; *iTRAQ*/MS-MS reagent, plasma sample
†LC-MS/MS method using an HPLC 1100 (Agilent, Applied Biosystems/Sciex API 2000 and a Waters XBridge Column; Sigma-Aldrich reagents; plasma samples)
LC-MS/MS method using a Waters Xevo TQ, Sigma-Aldrich reagents, and heparin-plasma samples from VWR

This study found that WA, HA, and WH values were significantly lower in malnourished children than in normal children. Our data correspond to those of Monika et al. (2010), who showed that out of 400 children living in Indian towns and villages, 90 children from villages and 10 children from towns exhibited wasting. Moreover, the nutritional status of town children is higher than that of village children [14]. Further, wasting was more frequent in male children compared with female children, which is consistent with the findings of Al-Mekhlafi et al., who found that male Malaysian children were more prone to experience wasting associated with parasitic hookworm infections [15].

In the present study, we found that the mean of ages of the malnourished and normal groups were not significantly different. For example, the mean values of body weight and height were lower in the malnourished group than in the normal group. The malnourished group had z-scores for WH and HA of less than –2 SD, and the z-score for WA was less than –3 SD. The nutritional status of the malnourished group indicates that wasting is characterized by significantly lower weight and shorter height. Most likely, the malnourished group was experiencing chronic malnutrition.

The concentrations of non-essential amino acids were not significantly different between the study groups, except for glycine and glutamic acid. These findings differ from those of Semba et al., who found that the concentrations of essential amino acids (tryptophan, isoleucine, leucine, valine, methionine, threonine, histidine, phenylalanine, and lysine), three conditionally essential amino acids (arginine, glycine, glutamine), three non-essential amino acids (asparagine, glutamic acid, serine), and citrulline differed significantly between stunted and normal children [16].

Essential amino acids cannot be synthesized by the body and must therefore be included in the diet. The following essential amino acids were examined in this study: leucine, methionine, phenylalanine, and arginine. The differences in the mean concentrations of valine, leucine, methionine, phenylalanine, and arginine of normal and malnourished children were not statistically significant.

Non-essential amino acids are synthesized by the body independent of diet. This study examined the following non-essential amino acids in this study: glycine, alanine, proline, ornithine, citrulline, tyrosine,

aspartic acid, and glutamic acid. The mean concentrations of alanine, proline, ornithine, citrulline, tyrosine, and aspartic acid were not significantly different between malnourished and normal children. In contrast, the mean glycine and glutamic acid concentrations were significantly higher in malnourished children than in normal children.

Glycine production can be maintained in malnourished individuals, because the simple structure of glycine can easily adapt to many conditions [17]. The relative increase in glutamic acid concentrations of malnourished undernutrition children may be caused by the reaction of α-ketoglutarate with aspartate to produce oxaloacetate and glutamate, which are involved in gluconeogenesis, glycolysis, and the citric acid cycle [18].

When we compared the mean values of amino acid concentrations of normal and malnourished children, most were consistent with the range of Malaysian reference values. The citrulline and arginine concentrations reported here are consistent with those reported by Morsy et al., who found normal concentrations of citrulline, ornithine, and arginine under conditions of severe energy deprivation caused by dietary protein deficiencies [19]. In this study, the concentration of glutamic acid in malnourished children was higher than the Singapore reference value, although the mean glycine concentration in normal children was lower than the lowest score of the Texas reference value. A comparison of our data with those of reference values determined in other countries suggests that further studies are required to establish a standard Indonesian amino acid concentration reference value.

5. Conclusion

Amino acid profiles of children with malnutrition did not significantly differ from those of normal children, except for the higher glycine and glutamic acid values in the malnutrition group.

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