

Profiles of Amino Acid in Indonesian Neonates

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Abstract

Background: Amino acid profiles in newborns is a sign of its nutritional status and it reflects the protein intake of the mother before and during pregnancy. The amino acid level is also a predictor of improved growth velocity and the only tool for diagnosis of amino acid disorder in suspected individuals. In Indonesia, based on National Basic Health Research year 2018, 48.8% of pregnant mother has anemia and 13% babies were underweight. Determining amino acid profiles is important to differentiate pathologic from normal condition in newborn population. There are only a few reports with adequate sample size on amino acid profiles in newborns from South East Asian Countries and none from the Indonesian population.

Methods: This is the first descriptive study in Indonesia newborns population determining the profiles of amino acid concentration from dried blood spot (DBS) sample by liquid chromatography-tandem-mass spectrometry (LC-MS/MS) system. This study used DBS sample obtained from the newborns' heel pricks, which is easier to store and handle in Indonesia's landscape. This will allow samples from remote area to be safely transported to referral laboratory.

Results: A total of 993 healthy newborns from 25 provinces and districts in Indonesia were included in this study. All samples were stored at -20oC and analyzed within 1 month. The amino acid concentration profile was summarized as 95% reference interval determined using nonparametric method. The result for most amino acid was only slightly different from previously reported reference from various population which was presumably caused by food preference. This study's result is expected to be implemented in Indonesian population.

Conclusions: Determining the amino acid profile in neonates using DBS is dependable. The result from this study is expected to be applied in our center and other referral hospital for inborn error of metabolism screening.

Background

Based on the report of the Indonesian National Basic Health Research year 2018, 48.8% of pregnant mother has anemia and 13% babies born were underweight. Severe and moderate malnutrition were observed in 3.9% and 13.8% children below 5 years old respectively and 29.9% cumulatively in children under 2 years old. Stunted growth were observed in 30.8% children under 5 years old.(1) Newborn babies get their protein from the mothers during gestation. The type of food ingested by and protein status of the mothers will affect the protein of their babies. Maternal nutrition especially protein intake plays an important role in the growth of the babies, low dietary protein intake can cause fetal growth restriction, low birth weight babies and also reduce the postnatal growth if there is certain amino acid deficiencies. (2)

Proteins are composed of amino acids chain, which are linked to one another by the peptide bond. Therefore, the body needs an adequate amount of amino acids to build a specific well-functioning

protein. These amino acids can be either synthesized by the body or ingested from the daily dietary intake or both.(3) Plasma concentration of amino acid has been regarded as one of the indicators of protein sufficiency while still taking into consideration the anthropometric and clinical data.(4) National data from 180 countries revealed that there was a relationship between protein quality, which is represented by its essential amino acid composition, total energy, digestibility, and stunting prevalence.(5, 6) Serum concentration of all essential amino acids and several non-essential amino acids had been found to be lower in stunted children.(6) For newborns, especially those with low and very low birth weight, plasma amino acid measurement can be useful as a means to monitor the efficacy of nutrient supply and a predictor to improved growth velocity.(7)

On the other hand, there is a subset of patients in which one or some of the amino acids accumulate in their body, resulting in a condition known as amino acid disorders or aminoacidopathies. This disorder is a subtype of inborn error of metabolism disease because the common basis pathogenesis of the disease includes an inherited defect in the metabolism pathway. In aminoacidopathies, the inherited mutation occurs in genes that determine the biological activity of an enzyme required for amino acid metabolism. In addition to accumulation of the upstream amino acids that fail to be metabolized, the blocked pathway activates alternative pathway, resulting in the production of certain metabolites that are not normally present.(8) Therefore, quantitative amino acid analysis is a critical test needed to detect these disorders in suspected infants and children.(9) The earlier the disorders are detected, the less severe and prevalent the complications caused by the abnormal accumulation of certain amino acid.

Based on those reasons, it is important to know the normal range of each amino acid concentration, especially the essential amino acids, in newborns. The quantitative method used for amino acid concentration measurement utilizes liquid chromatography and spectrophotometry. There are two types of sample that can be processed for the measurement: the plasma and dried blood spot. While plasma amino acid concentration has been held as the benchmark, it requires 2–4 mL of whole blood and needs to be stored at the minimum temperature of -20°C or even -80°C for it to be used in the maximum period of 15 and 30 days, respectively.(10–12) On the other hand, the volume of whole blood needed for dried blood spot (DBS) sample can be as little as 12–24 μL . The filter paper containing the DBS can be stored at either room temperature or as low as 4°C as long as the environment is kept dry.(13) However, there exists only limited reports with adequate sample size on amino acid concentration profile in newborns obtained from DBS sample in South East Asian Countries and none for the Indonesian population.(12, 14)

Methods

Study Design and Setting

The aim of this study was to determine the profile of amino acid concentration from DBS sample by liquid chromatography-tandem-mass spectrometry (LC-MS/MS) system in Indonesia newborns population. This is a cross-sectional descriptive study conducted at Dr. Cipto Mangunkusumo Hospital as

the National Referral Hospital in Indonesia from November 2018 to November 2019 and Indonesian Institute for Medical Education and Research Faculty of Medicine University of Indonesia. The samples were taken from newborn babies in the neonatal ward of hospitals and primary care clinics from 25 provinces and districts in Indonesia. The informed consent was given by the parents. This research was approved by the Dr. Cipto Mangunkusumo Hospital Ethics Committee (No.LB.02.02/22/144/2018).

Study Population

A total of 993 DBS samples were included in this study. Samples were sent for hypothyroid screening, and the DBS samples included in this study were screened using several inclusion and exclusion criteria. The inclusion criteria were: term newborns, with normal birth weight (2500–3500 g) appropriate for the gestational age, and normal TSH level. The exclusion criteria were if the DBS samples quality were too small or compromised with mould.(12, 15)

Sample Collection

The blood collection was collected between day 1 to 7 after birth. The blood was taken from the heel of the newborn using a lancet with a tip approximately 2 mm and dripped onto a filter paper (Whatman 903) in the designated 13-mm circle. This would result in the collection of 80–100 uL blood for each circle. The blood spots were air dried on a dry, even, and non-absorptive surface at 20 to 25°C and sent to Dr. Cipto Mangunkusumo Hospital for hypothyroid screening. After DBS samples were taken for the screening, the rest of the DBS samples were stored at -20°C until they were analyzed for amino acids.(12, 14)

Amino Acid Analysis

Analysis was performed by LC-MS/MS system (Xevo TQD Tandem, Triple Quadrupole Mass Spectrometer; Waters Corporation, Massachusetts, USA). The protocol for amino acid measurement from DBS was in compliance with the manufacturer's guideline (Chromsystems, Munich/Germany and ClinSpot, Munich/Germany), as follows. A 3 mm DBS disk was punched out of the filter card into a 96 well plate. A total of 100 uL reconstituted internal standard was added and the 96 well plate was sealed with a protective sheet. The plate was then agitated at 600 rpm for 20 minutes at room temperature. The supernatant was transferred into a new 96 well plate and evaporated at 40 °C for 30 minutes (ClinSpot) or 60°C (Chromsystem) to complete dryness. For derivatization, a 50 uL of n-Butylester was added and then the plate was sealed with a pierceable adhesive before incubated for 20 minutes at 60°C. Afterwards, the sample was again evaporated to complete dryness. Reconstitution steps encompassed of adding 100 ul of reconstitution buffer and resealed before agitated at 700 rpm for 5 minutes. The supernatant was injected into the LC-MS/MS system with a total volume of 10 uL. The HPLC pump was set at a constant flow rate of 20 to 600 uL/min with 1.7 minutes run time. After electrospray ionization and transfer to a gas phase, samples were relayed into the MS/MS system and analytes measurement was carried out in multiple reaction monitoring (MRM) mode.(16, 17)

Data Analysis

Statistical analysis was performed using SPSS 20.0 (IBM, New York). In this study we recapitulated the amino acid concentration profile as 95% reference interval using the nonparametric statistical method. (18, 19) We identified and excluded outliers in a two-stage process if the data is not distributed normally. First, we transformed the data so the distribution resembled the Gaussian population using Box and Cox method or modulus function for skewness or kurtosis correction, respectively.(18, 20, 21) Secondly, we used a Tukey's method for outlier detection. This method sets computed upper and lower limits for exclusion. The upper limit is the 75th percentile value plus 1.5 times interquartile range while the lower limit is the 25th percentile value minus 1.5 times interquartile range. All values outside this limit are considered as outliers.(20) The two-stage outlier detection scheme attempts to balance both the underestimation and overestimation if outliers were included and excluded, respectively.(18)

Results

For quality control, we conducted 5 times amino acid measurement of control with known concentration. The intraassay coefficient of variation (CV) for each amino acid is less than 10% except for aspartic acid (CV 10,73) and with the lowest value was 1.88% for leucine. (Table 1).(16, 22)

The profile of thirteen amino acids from our study was shown in Table 3. We also compared our results to reference interval from previous reports. (12, 14, 23–25)

Table 1
Coefficient of variation measurement.

Amino Acid	CV		
	This research	ChromSystem(16)	ThermoFisher Scientific(22)
Glycine	2,05	5.0–10.9	6.8–8.8
Alanine	4,34	4.6–5.2	8.9–11.5
Proline	2,87	5.4–8.8	na
Valine	2,65	2.3–4.8	8.1–9.6
Leucine	2,25	2.8–6.4	6.8–8.5
Ornithine	1,42	5.4–7.1	8.4–12.3
Methionine	6,25	2.9–6.8	6.4–8.7
Phenylalanine	2,26	3.0–4.7	5.5–8.5
Arginine	5,73	3.4–10.4	5.4–9.6
Citrulline	6,11	5.0–7.3	4.0–6.8
Tyrosine	3,69	2.9–4.7	7.8–10.8
Aspartic acid	10,73	4.7–5.8	6.5–9.0
Glutamic Acid	1,53	3.0–4.9	5.9–10.1

Discussion

This is the first study to analyze the profiles of amino acid in newborns in Indonesia. The first 1000 days of life (i.e. from conception until 2 years old) is considered as the critical time for human development. Analyzing the profile of amino acid in newborn showed the influence of the nutritional status and protein nutrients taken by the mothers during gestation.(1) Indonesian pregnant women in different provincial area showed different preferences of food as there are many subethnicity in Indonesia. This study did not differ the gender of the babies, Bergwerff et al did not find any differences between genders.(26) Our eligibility criteria in this study could exclude most if not all unhealthy infant conditions that could have deviated the study population characteristic, especially the plasma amino acid concentration.(15) The samples also did not include babies with abnormal thyroid function or low birth weight babies, in order to exclude patients with conditions that might otherwise affect the study outcomes. We selectively screened neonates to make sure that they are healthy before enrolling them into the study.(20, 27)

This study included 993 subjects, a number which exceeds the minimum sample for reference interval study (120 samples), as recommended by the Clinical Laboratory and Standard Institute (CLSI) and this results might be used by clinican as a reference to detect other abnormality in amino acids.(27) Even

though the newborn's age group is considered the most challenging age group to obtain sample from, we succeeded to fulfil statistical sufficiency on number of subjects in this study.

As depicted in Table 2, we compared our finding with results from previous studies. Arginine range in study using DBS, including our current study, was found to have decreased lower and upper limit compared to result from studies using plasma as sample. A study by Wuyts et al found that measurement of amino acids participating in urea cycle metabolism such as arginine, citrulline, and ornithine were affected by pH level during extraction and elution time when it is from DBS sample.(28) It is recommended to set the pH at lower level around 2–3 for arginine analysis. In our study, elution pH was set at 3 based on kit recommendation. Therefore, pH was unlikely to be a matter of low arginine measurement in our study. Difference in nutrient intakes during pregnancy in Indonesian women must be accounted for low arginine. Further study regarding diets on Indonesian mother needs to be done.

Table 2
Comparison of amino acid reference intervals in newborn.

Amino acid (umol/L)	This research Median (Lower-Upper) [†]	USA (NCS cohort) [‡]	Thailand [§]	USA (Mayo Clinic and University of Iowa) [¶]	USA (OU Medicine) ^{††}	Canada ^{‡‡}
Glycine	395.95 (186.17–919.2)	329 (182–637)	345 (300–414)	111–426	232–740	299–782
Alanine	236.16 (110.85–608.44)	188 (104–394)	543 (424–633)	139–474	131–710	175–427
Proline	172.83 (93.68–363.58)	155 (94–245)	122 (97–150)	85–303	110–417	127–292
Valine	95.93 (41.39–249.47)	95 (46–224)	112 (89.1–179.2)	83–300	86–190	87–326
Leucin	139.99 (58.52–433.36)	60 (31–130)	602 (511–703)	48–175	48–160	46–165
Ornithine	161.95 (44.35–703.98)	45 (19–105)	151 (124–185)	20–130	48–211	82–365
Methionine	7.01 (1.66–34.22)	21 (10–39)	14.1 (10.7–17.7)	11–35	10–60	13–44
Phenylalanine	51.69 (29.9–99.68)	51 (30–97)	59 (50.3–70.1)	28–80	38–137	49–107
Arginine	9.04 (0.71–53)	9 (< 1–36)	8.4 (5.6–14.7)	29–134	6–140	2–118

Amino acid (umol/L)	This research Median (Lower-Upper) [†]	USA (NCS cohort) [‡]	Thailand [§]	USA (Mayo Clinic and University of Iowa) [¶]	USA (OU Medicine) ^{††}	Canada ^{‡‡}
Citrulline	31.07 (4.33–139.02)	12 (5–23)	16.9 (13.8–23)	9–38	10–45	9–44
Tyrosine	105.45 (53.8–203.03)	72 (34–151)	91.1 (74.1–114.4)	26–115	55–147	27–187
Aspartic acid	26.62 (10.68–75.22)	N/A	N/A	2–20	20–129	19–121
Glutamic acid	572.59 (243.39–1221.2)	324 (193–566)	N/A	31–202	62–620	91–401

[†]Reference values from this study

[‡]Reference values from National Children Study's cohort Canada; LC-MS/MS system (Agilent, Santa Clara, USA and Applied Biosystem, Foster City, USA); Dried blood spot. NCS: National Children's Study. (1)

[§]Reference values from King Chulalongkorn Memorial Hospital Thailand; LC-MS/MS system (Waters, Milford, USA); Dried blood spot.(2)

[¶]Reference values from Mayo Clinic Laboratory USA; LC-MS/MS system (Quest Diagnostic, USA); Plasma.(3)

^{††}Reference values from OU Medicine, Oklahoma, USA; LC-Ninhydrin; Plasma.(4)

^{‡‡}Reference values from The Hospital for Sick Children, Canada; LC-MS/MS system; Plasma.(5)

References for table

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Amino acid (umol/L)	This research Median (Lower-Upper) [†]	USA (NCS cohort) [‡]	Thailand [§]	USA (Mayo Clinic and University of Iowa) [¶]	USA (OU Medicine) ^{††}	Canada ^{##}
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Among studies using DBS sample, there were several amino acids whose plasma level were discernibly different. Alanine level in our study resembled the finding in all other studies.(14, 23–25) However, in the Thailand study the alanine level was markedly increased.(12) Eight percent of amino acids in all human proteins is alanine. Therefore, when an increased rate of proteolysis happened (e.g. muscle proteolysis due to impaired glucose oxidation secondary to insulin resistance), alanine plasma level would be increased.(29) Alanine is also the major amino acid source in the gluconeogenesis pathway in human in which it will be converted into pyruvate. Any deficiency in this pathway will also cause increased alanine level in blood.(30) Other than that, high alanine level was also found to be related to overfeeding especially in individuals with decreased insulin sensitivity.(29) All the newborns in Thailand study received breast milk with normal Z score for weight/age, weight/height, and body mass index (BMI) while neither our or the Canadian study specified the feeding status of our subjects. Leucine level in the Thailand study was also noticeably higher although our study also found a higher upper limit than other studies, regardless the sample type. Lower leucine level had been shown to be the biochemical marker of protein economy due to rapid growth. However, this would only be apparent after a few weeks after birth. (31) Serum leucine concentration in infants reflect their oral leucine intake in a linear fashion and milk-derived leucine intake of pregnant mother is in turn correlated with increase of the infant's birth weight. Therefore, the higher leucine level in newborns of certain population may indicate both the newborns' and the mother's leucine intake during the breastfeeding and pregnancy.(32) The possibility that different leucine level amongst studies resulted from ethnicity difference cannot be excluded yet as there is no previous report investigated this in newborn population.

Ornithine level in our study resembles the Thailand study and its median is close to other studies using plasma samples. However, it is approximately four times higher than the one from NCS cohort and its upper limit is also markedly higher among all other studies. Ornithine, arginine, and citrulline are amino acids involved in urea cycle, in addition to being a building block of protein. Several conditions resulting in protein breakdown and hence an increased urea excretion (e.g. protein-rich diet, starvation, exogenous

corticosteroids) are associated with elevated urea cycle enzymes and led to urea synthesis. However, the hepatocytes where this cycle mainly takes place maintain the steady state, intracellular level of all three amino acids, despite increased enzyme and urea level.(33) One pathologic condition affecting plasma level of these amino acids are short bowel patients. The small intestines convert glutamine to citrulline and it is then converted to arginine. The glutamine level in short-bowel patients was significantly higher while both citrulline and arginine level is lower. In newborns, although the diet is usually deficient in arginine, the glucose metabolism via pentose pathway indirectly supports arginine synthesis from glutamine.(34) This is in accordance with the finding in one study that revealed no association between diet and plasma levels of ornithine, arginine, and citrulline.(33) Mature neonates have significantly higher plasma level of arginine and citrulline compared with preterm neonates.(35) However since neonates included in each of the study were term, this was an unlikely explanation of the difference of the amino acid levels among the studies. Instead, the discrepancies might have arisen either from different level of enzymatic activities affected by ethnic background or from technical reasons, as amino acids involved in urea cycle are susceptible to the environment condition of extraction.

Other amino acid whose plasma level overtly differ from other studies is methionine. In our study, the median was 2 times lower than any other study although the reference range is close to the others. An animal study found that maternal consumption with higher methionine levels (either as DL-methionine or DL-2-hydroxy-4-methylthiobutanoic acid) resulted in higher plasma methionine level in the offspring.(36) As maternal dietary record and the time at which the DBS sample taken in this study were not evaluated, it cannot be concluded that the lower methionine level found in this study was indeed due to inadequacy of methionine and total energy in the diet.

Glutamic acid is the only amino acid with highly variable measurement amongst all studies in comparison. This amino acid can be derived from either glutamine or Kreb's cycle intermediates. The conversion of glutamine to glutamic acid is bioenergetically favorable while the opposite requires glutamine synthetase enzyme. On the other hand, conversion from α -ketoglutarate to glutamic acid is dependent to glutamate dehydrogenase.(37) Consequently, glutamic acid level is subject to change when activity of these enzymes is affected by either external or internal factor such as genetic polymorphism. Moreover, glutamic acid is also present ubiquitously in many foods.(37)

Tyrosine plasma level in newborns are highly affected by the amount of protein in the diet, vitamin C level, and maturation of the enzyme 4-hydroxyphenylpyruvate dioxygenase (4HPPD) in the liver. High protein diet along with vitamin C deficiency usually cause benign, transient increased level of tyrosine in the newborn. Furthermore, the maturation of 4HPPD enzyme is dependent on the gestational age: a one-week difference, although both are at term, can result in the enzyme's different level of function.(38, 39) Our finding might have been caused by these factors, in addition to laboratory techniques.

In this study two different kits were utilized to measure the amino acid level. Both kits had an agreeable intra- and interassay precision which was represented by the coefficient of variance (CV) of less than 10% and 15%, respectively.(16, 17) Nevertheless, different population, methodology, appliances, and laboratory

environment could have affected the result of this study and caused the disagreement between our and previous studies.

Conclusion

Amino acid analysis using LC-MS/MS from DBS sample can be done in short period of time with sufficient precision. The advantages of using DBS sample include easier retrieval, especially in at term, healthy newborns in whom laboratory examination is not indicated, and lesser volume than the plasma specimen. In a rural area, DBS sample can be transported with an ease to referral center for suspicion of pathologic condition such as inborn error of metabolism. The amino acid value from this study is expected to be applied in our center and other referral hospital for management of newborns with abnormal protein status, especially for their dietary recommendations.

Abbreviations

DBS
dried blood spot
LC-MS/MS
liquid chromatography-tandem-mass spectrometry

Declarations

- Ethic approval and consent to participate

This research had been granted ethical approval by the Dr. Cipto Mangunkusumo Hospital Ethics Committee (No.LB.02.02/22/144/2018). The parents of newborns fulfilling research criteria were informed about the study and asked for their consent for the patients to be included in the study.

- Consent for publication

Not applicable

- Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

- Competing interest

The authors declare that they have no competing interests

- Funding

The research was funded by Research Funding Universitas Indonesia. The funding body did not participate in determining the study design, data collection, analysis, and interpretation, and in manuscript writing.

- Authors' contribution

Dr. dr. Ina Susianti Timan, SpPK(K), Dr. dr. Merci Monica Pasaribu, SpPK, dr. Latifah Anandari, SpPK: Designing the research, conducting the measurements in research, and data analysis

Prof. Dr. dr. Damayanti Rusli Sjarif, SpA(K): Designing the research, subject recruitment, and data analysis

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1. Made Ananda Krisna for contribution in statistical calculation

Conflict of interest

None declared

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