Normal Plasma Free Amino Acid Levels in Thai Children

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Abstract

Analysis of plasma free amino acid levels is important for diagnosis of inborn errors of metabolism. Traditionally, this is performed using commercially available dedicated amino acid analyzers, but few such instruments are available in Thailand, and many are not used in routine operations. Here, the authors describe the analysis of plasma free amino acid levels in 57 normal children by reverse-phase HPLC and pre-column derivatization with phenylisothiocyanate. Plasma free amino levels are reported as mean ± SD and 95 per cent confidence interval of mean for each of 5 age groups: 0-6 months; 6-12 months; 1-3 years; 3-6 years; 6-12 years. Mean amino acid levels were generally similar in all age groups ($p \geq 0.01$), except that hydroxyproline tended to be higher in the 0-6 months age group compared to other age groups ($p < 0.01$).

Comparisons were made between the present data with the normal free plasma amino acid levels in children of similar age groups reported both in Thailand and overseas in terms of both mean ± SD and maximum and minimum values. Overall, our methodology involving HPLC can identify 35 amino acid derivatives, including all the major amino acids except for cysteine, which is substantially more than the number reported in earlier work on plasma free amino acid levels in normal Thai children. Moreover, the present methodology gives mean ± SD values similar to an overseas report. For these reasons, HPLC should be considered as an alternative approach in laboratories, where demand does not justify the need for dedicated amino acid analyzers. However, there can be substantial variations between the results from different laboratories, and each laboratory should establish its own normal values.

Key word : Amino Acid, Inborn Error, Plasma

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Quantitation of amino acids in physiological fluids is essential for the evaluation of inborn errors of metabolism[1]. Although the major causes of hospitalized infants usually derive from sepsis or disorders of respiratory or cardiovascular systems, a reasonable proportion are due to a group of biochemical genetic disorders or inborn errors of metabolism. Several catastrophic illnesses, including brain damage and mental retardation result from excess or depletion of certain amino acids in plasma. Abnormal levels of amino acids can give rise to serious problems, e.g. elevation of phenylalanine results in phenylketonuria[2] and elevation of branched-chain amino acids (such as leucine, isoleucine and valine) results in maple syrup urine disease[1,3]. Many inborn errors of metabolism have been described in Thailand[4], many of which are due to defects in the metabolism of amino acid derivatives. Ideally, inborn errors of metabolism should be detected as early as possible, so that suitable treatment may be administered. Accordingly, medical practitioners need to know what the normal values of plasma amino acids in infants and children are.

Plasma amino acid levels for normal infants and children are available in Western textbooks[5-10]. There is much less data on plasma amino acid levels in normal Thai people. Suvanapha et al[11] investigated the plasma amino acid levels in 22 normal Thai adults and in 43 Thai patients with renal failure. After the present work was started, Sirichakwal et al[12] reported the plasma amino acid levels of 63 children and 73 adults, but this was actually work that had been performed many years ago, but had hitherto not been published. Moreover, both the Thai studies were performed using dedicated amino acid analyzers, which are not so readily available in Thailand. The authors, therefore, wish to report the determination of normal plasma amino acid levels of 57 Thai infants and children of different age groups, using high-performance liquid chromatography (HPLC), which is more widely available in Thailand.

MATERIAL AND METHOD

Blood samples were collected from infants and children briefly hospitalized for minor illnesses, e.g. gastroenteritis, pneumonitis, and dehydration. The selected subjects were basically in good health prior to their hospitalization, and were chosen with the following criteria: a) no history of liver or kidney disease, and having normal liver and kidney function tests; b) no evidence of malnutrition; c) normal growth (height between 10-90 percentile and head circumference between 10-90 percentile) and normal developmental milestones; d) on a regular diet intake.

Heparinized blood samples were collected in a centrifuge tube and immediately separated by centrifugation at 1,200 rpm, 4°C for 7 min. The supernatant was used for amino acid analysis using the Waters Picotag™ system for biological fluids [13]. Samples were prepared following the protocol described by Matteson[14]. 60 µl of plasma was mixed with 10 µl of 0.4 mM methionine sulfone (as internal standard) in 0.1 M HCl, made up to 300 µl, and then deproteinized in a Lida microspin filter unit (MW cut off 10,000 daltons). An aliquot of the filtrate (50 µl) was derivatized with phenylisothiocyanate, dried under vacuum and resuspended in 100 µl Picotag™ sample diluent. Amino acids were separated on a Picotag™ column (3.9 × 300 cm) in a Waters 510 HPLC (Milford, MA, USA). Separation of the PTC (phenylthiocarbamyl) derivatives of amino acids was performed using a gradient of 0.067 M sodium acetate buffer, pH 6.5:acetonitrile (97.5:2.5) (buffer A) and acetonitrile:methanol:water 45:15:40 (buffer B) at 46°C, according to the program: 0 min 100%A:0%B; 13.5 min 97%A:3%B; 24 min 94%A:6%B; 30 min 91%A:9%B; 50 min 66%A:34%B; 62 min 0%A:100%B; 62.5 min 0%A:100%B; 66.5 min 100%A:0%B; 67 min 100%A:0%B; 87 min 100%A:0%B. PTC-amino acids were detected at 254 nm, and were quantitated using standard mixtures containing 500 pmoles/amino acid.

A total of 57 samples (25 males and 32 females) were collected at the Department of Pediatrics, Faculty of Medicine Siriraj Hospital, Mahidol University and at the Queen Sirikit National Institute of Child Health, Ministry of Public Health. The 57 samples were divided into five age groups as follows: group 1, 0-6 months (m) (n=15); group 2, 6-12 m (n=10); group 3, 1-3 years (yr) (n=10); group 4, 3-6 yr (n=11); group 5, 6-12 yr. Plasma amino acid concentrations were expressed as mean, standard deviation, and 95 per cent confidence interval values. Comparisons among age groups were made with one way analysis of variance with Levene statistic and t-statistic; $p<0.05$ or $p<0.01$ was taken to indicate statistically significant difference. In the case of heterogeneity of variances, t-statistic was used to investigate the significance of differences between the means of different age groups. Further-

more, t-statistic was also employed to compare the present studies with other studies. Data was analyzed by the statistical package, SPSS for Windows version 7.5.

RESULTS

Good separation of PTC-amino acids was obtained by HPLC, and 35 amino acid derivatives could be quantitated, using methionine sulfone as an internal standard. Plasma amino acid levels in children are shown as mean, SD, and upper and lower values at 95 per cent confidence intervals from mean (95% CI) for the different age groups (Table 1). Since the levels of each amino acid in plasma showed no significant differences between males (n=25) and females (n=32) at $p<0.05$, the data for males and females are considered together. Although mean values are useful for the purposes of comparison, the 95 per cent CI values will be more useful to the clinician in deciding whether to perform more detailed follow-up studies on the patient.

The plasma levels of most amino acids were similar in the different age groups. However, analysis of variance of each amino acid between different age groups was performed using the F-test and the Levene test for equality of variance. Where Levene statistics gave $p<0.05$, multiple comparisons were made using the *t*-test. Only the comparisons giving $p<0.05$ in the *t*-test are shown in Table 2. Four amino acids showed $p<0.05$ in age group comparisons, with citrulline being lower in groups 2 and 3 compared to group 4, threonine being higher in group 1 compared to groups 3 and 4, tyrosine being higher in group 1 than in group 3, and methionine being higher in group 1 compared to group 2 and 3. However, most notable among the amino acids was hydroxyproline, which was significantly higher ($p<0.01$) in group 1 than in groups 3, 4 and 5, and also showed differences between group 3 and group 4/group 5 ($p<0.05$).

Comparisons were also made with other selected data on the plasma amino acid levels in infants and children reported both locally and overseas. However, there is much variability between different reports, both in the ranges of ages studied and in the way data are reported, since some authors express data in the form of mean values, while others report data as maximum and minimum values or values at 95% CI. Statistical comparisons of mean ± SD are made between our data for age group 1 (1 yr-3 yr) with data of Sirichakwal et al[12] for the same age group (1 yr-3 yr), data from France[8] for the age group 10 m-3 yr and data from the USA [7] in the age group 1 yr-5 yr. The number of amino acids analyzed and reported differ in the four studies. So, only data for the 20 amino acids reported by all four groups are listed and compared in Table 3. There are substantial variations between the groups, but this did not appear to be due to any intrinsic differences between Thai and Western children. Thus, our data (A) differs from the data of Sirichakwal (B) and the data of Meites (D) at $p<0.05$ level in 12 and 13 amino acids respectively, but differ from that of Ghisolfi (C) in only 6 amino acids. The data of Sirichakwal et al (B) differ from that of Meites et al (D) in only 5 amino acids, but differ from the data of Ghisolfi et al (C) in 14 amino acids. Finally, the data of Ghisolfi et al (C) differ from the data of Meites et al (D) in as many as 15 amino acids.

The maximum and minimum values observed in our study were also compared to the data of Sirichakwal et al[12], and the data from two standard textbooks, namely those of Shapira et al [5] and Behrman et al[6] in Table 4. The data of Shapira et al[5] list as many as 40 amino acids which could be analyzed, although four of these were listed as being present at a level of 0 μM. Our data list 35 amino acids, which could be resolved and analyzed, although seven were present in levels too low to quantitate and are shown as <5 μM or <10 μM. On the other hand, the data of Behrman et al[6] list 29 amino acids, while the data of Sirichakwal et al[12] list only 21 amino acids.

DISCUSSION

There have been many developments in the methodology for determination of amino acids. The automated amino acid analyzer[15], based on a two column ion-exchange chromatographic separation of amino acids, followed by quantitation by reaction with ninhydrin was improved into a single column system[16], which reduced the amount of sample as well as errors from dividing samples into parts. Introduction of HPLC methods further increased speed and sensitivity[17], which was followed by dramatic advances in the electronic industry, leading to improved automation. With developments over the last 30 years, the modern amino acid analyzer, employing ion-exchange chromatography followed by post-column detection with ninhydrin, has

Table 1. Mean, SD, and 95 per cent confidence interval of plasma amino acid levels for different age groups.

Amino acids	Group 1 (0-6 months)				Group 2 (6-12 months)				Group 3 (1-3 years)			
	Mean	SD	95% CI		Mean	SD	95% CI		Mean	SD	95% CI	
	(n = 15)		Lower	Upper	(n=10)		Lower	Upper	(n=10)		Lower	Upper
Phosphoserine	18.9	11.4	12.6	25.3	23.5	26.7	4.4	42.6	14.8	11.1	6.8	22.7
Aspartic acid	8.0	4.9	5.2	10.7	8.1	6.3	3.6	12.7	4.3	1.7	3.0	5.6
Glutamic acid	199.9	137.5	123.7	276.0	218.8	201.5	74.6	363.0	144.3	133.1	49.0	239.5
α-Aminoadipic acid	2.1	2.6	0.6	3.5	1.3	1.7	0.0	2.6	1.3	1.5	0.2	2.4
Hydroxyproline	28.9	13.6	21.3	36.5	17.6	18.5	4.4	30.9	8.0	2.6	6.1	9.9
Phosphoethanolamine	0.5	1.8	0.0	1.6	1.3	2.3	0.0	3.0	1.2	2.4	0.0	2.9
Serine	130.9	56.4	99.6	162.2	116.1	46.9	82.5	149.7	105.6	42.9	74.9	136.3
Asparagine	84.2	24.2	70.8	97.7	72.8	29.6	51.6	94.0	67.4	24.8	49.6	85.2
Glycine	191.1	90.5	141.0	241.3	158.7	52.9	120.8	196.5	146.7	47.5	112.7	180.8
Glutamine	421.9	197.3	312.7	531.2	317.0	207.2	168.8	465.2	268.0	116.7	184.5	351.5
β-Alanine	4.4	2.4	3.1	5.8	2.9	2.5	1.2	4.7	4.8	3.8	2.1	7.6
Taurine	96.1	57.5	64.2	128.0	143.7	102.9	70.0	217.4	80.6	54.9	41.4	119.9
Histidine	27.0	17.5	17.2	36.7	26.8	10.1	19.6	34.1	16.7	11.9	8.1	25.3
γ-Aminobutyric acid	<10				<10				<10			
Citrulline	33.7	35.8	13.9	53.6	18.1	13.3	0.7	42.7	17.8	12.8	8.6	27.0
Threonine	135.9	104.3	78.1	193.7	82.2	59.6	36.4	128.0	59.5	40.9	30.2	88.8
Alanine	278.7	101.6	222.4	334.9	222.6	123.9	133.9	311.3	197.6	84.8	136.9	258.3
β-Amino-isobutyric acid	<5				<5				<5			
Carnosine	<5				<5				<5			
Arginine	66.0	30.7	47.4	84.6	78.9	39.4	50.8	107.1	56.3	33.1	32.6	80.0
Proline	198.3	73.8	157.5	239.2	184.8	100.9	112.6	257.0	113.1	60.4	69.9	156.4
1-Methylhistidine	<5				<5				<5			
Anserine	<5				<5				<5			
3- Methylhistidine	<5				<5				<5			
α-Amino-n-butyric acid	13.3	12.8	6.2	20.4	16.3	8.7	10.0	22.5	12.8	6.8	7.9	17.8
Tyrosine	72.9	29.9	56.3	89.4	55.8	33.1	32.1	79.5	44.7	16.5	32.9	56.5
Valine	160.3	53.7	130.5	190.1	162.5	64.1	116.7	208.4	158.3	72.9	106.1	210.5
Methionine	32.0	11.5	25.6	38.4	19.7	10.1	12.5	27.0	19.6	9.2	13.0	26.3
Isoleucine	44.3	16.4	35.1	53.4	45.1	21.6	29.6	60.6	40.5	20.5	25.8	55.2
Leucine	91.1	27.2	76.0	106.2	99.3	46.1	66.3	132.3	95.8	47.6	61.7	129.8
Hydroxylysine	<5				<5				<5			
Phenylalanine	53.6	13.3	46.2	61.0	52.8	13.3	43.2	62.3	59.4	13.4	49.8	69.0
Tryptophan	16.8	7.4	12.7	20.9	16.1	8.7	9.9	22.3	13.0	5.5	9.0	17.0
Ornithine	53.1	32.8	34.9	71.3	40.9	25.1	22.9	58.9	31.7	20.8	16.8	46.7
Lysine	109.6	55.1	79.1	140.2	106.9	54.4	68.0	145.9	93.8	60.7	50.3	137.2

Table 1. (continued)

Amino acids	Group 4 (3-6 years)				Group 5 (6-12 years)				Total (0-12 years)			
	Mean	SD	95% CI		Mean	SD	95% CI		Mean	SD	95% CI	
	(n = 11)		Lower	Upper	(n=11)		Lower	Upper	(n=57)		Lower	Upper
Phosphoserine	22.6	39.4	0	49.1	18.2	7.5	13.2	23.3	19.6	21.5	13.9	25.3
Aspartic acid	5.8	5.4	2.1	9.4	7.0	5.2	3.5	10.5	6.8	5.0	5.4	8.1
Glutamic acid	124.3	64.2	81.1	167.4	160.7	150.0	63.3	258.1	171.3	140.7	134.0	208.7
α-Aminoadipic acid	2.4	4.0	0	5.1	1.1	1.1	0.3	1.8	1.7	2.5	1.0	2.3
Hydroxyproline	14.1	6.9	9.4	18.7	16.9	7.2	12.1	21.7	18.1	13.2	14.6	21.6
Phosphoethanolamine	2.4	3.6	0	4.8	2.9	5.0	0	6.3	1.6	3.2	0.8	2.5
Serine	115.4	37.3	90.3	140.4	108.9	36.9	84.1	133.7	116.6	45.0	104.7	128.6
Asparagine	86.4	31.6	65.1	107.6	89.3	24.5	72.8	105.8	80.7	27.2	73.5	87.9
Glycine	202.4	80.3	148.4	256.4	187.4	48.3	154.9	219.9	179.1	69.6	160.6	197.6
Glutamine	377.0	133.4	287.3	466.6	347.5	124.7	263.7	431.3	353.5	166.4	309.3	397.6
β-Alanine	6.0	3.8	3.4	8.6	7.7	5.9	3.7	11.6	5.2	4.0	4.1	6.2
Taurine	124.1	60.4	83.5	164.8	125.8	101.2	57.8	193.9	112.9	77.4	92.3	133.4
Histidine	21.7	11.2	14.2	29.3	20.3	15.1	10.1	30.6	22.9	14.0	19.1	26.6
γ-Aminobutyric acid	<10				<10				<10			
Citrulline	60.5	50.7	26.4	94.5	37.2	28.6	18.0	56.5	34.0	34.8	24.8	43.3
Threonine	58.3	34.4	35.1	81.4	80.3	42.6	51.6	108.9	87.5	71.2	68.4	106.5
Alanine	283.1	136.9	191.1	375.1	332.3	127.0	246.3	417.6	265.8	120.7	233.8	297.8
β-Amino-isobutyric acid	<5				<5				<5			
Carnosine	<5				<5				<5			
Arginine	73.1	45.5	42.5	103.6	79.9	27.8	61.2	98.6	70.8	35.4	61.2	80.3
Proline	189.4	111.5	114.5	264.4	163.9	67.8	118.3	209.4	172.6	86.8	149.6	195.7
1-Methylhistidine	<5				<5				<5			
Anserine	<5				<5				<5			
3- Methylhistidine	<5				<5				<5			
α-Amino-n-butyric acid	20.3	13.3	11.4	29.3	15.9	9.4	9.6	22.2	15.6	10.7	12.7	18.5
Tyrosine	55.1	26.0	37.6	72.6	116.0	113.0	40.1	192.0	69.8	58.8	54.2	85.5
Valine	177.2	59.2	137.4	217.1	210.6	66.6	165.9	255.4	173.3	63.5	156.4	190.2
Methionine	25.0	12.7	16.5	33.6	26.7	9.5	20.2	33.1	25.3	11.5	22.2	28.4
Isoleucine	48.3	17.1	36.8	59.8	51.9	21.3	37.5	66.2	46.0	18.9	41.0	51.0
Leucine	97.3	31.5	76.1	118.5	103.4	24.6	86.8	120.0	96.9	34.5	97.8	106.1
Hydroxylysine	<5				<5				<5			
Phenylalanine	57.8	13.5	48.7	66.9	63.1	19.7	49.8	76.3	57.1	14.7	53.2	61.0
Tryptophan	14.9	7.1	10.1	19.7	19.2	9.5	12.8	25.6	16.1	7.7	14.0	18.2
Ornithine	41.3	15.9	30.6	52.0	48.4	15.5	37.6	58.8	44.0	24.2	37.6	50.4
Lysine	112.6	39.0	86.3	138.8	128.2	41.9	98.2	158.1	110.2	50.4	96.7	123.7

Table 2. Comparison of plasma amino acids for different age groups with t and p-value.

Amino acid compounds	Age groups		t	p-value
Hydroxyproline:	0-6 months	1-3 years	5.764**	0.000
		3-6 years	3.629**	0.001
		6-12 years	2.910**	0.008
	1-3 years	3-6 years	-2.672*	0.019
		6-12 years	-3.827**	0.002
Citrulline:	6-12 months	3-6 years	-2.668*	0.021
	1-3 years	3-6 years	-2.698*	0.020
Threonine:	0-6 months	1-3 years	2.556*	0.019
		3-6 years	2.688*	0.015
Tyrosine:	0-6 months	1-3 years	2.702*	0.013
Methionine:	0-6 months	6-12 months	2.720*	0.012
		1-3 years	2.823*	0.010

* significant at p-value <0.05
** significant at p-value <0.01

evolved into a highly accurate and sensitive instrument[18]. At the same time, various alternative approaches for analyzing amino acids have been developed using gas-liquid chromatography with pre-column dramatization[19] and reversed HPLC with pre- or post-column derivatization[20-22], which are also sensitive and reliable.

Dedicated amino acid analyzers are commercially available from a number of companies. However, as dedicated instruments, they are limited in their use to the sole function of analyzing amino acids, so that this makes them uneconomical in laboratories which have a limited number of samples. Indeed, in a developing country such as Thailand, few laboratories can afford to purchase instrumentation for determining amino acids alone. Moreover, amino acid analyzers are sophisticated instruments, which have to be properly maintained and operated to function properly. Our laboratory at the Chulabhorn Research Institute started using reversed-phase HPLC and pre-column derivatization with PITC for determining the amino acid composition of peptide and protein hydrolyzates more than 10 years ago. Then, after expanding our research into inborn errors of metabolism, we found that physicians in this field had considerable difficulties obtaining access to amino acid analyzer facilities locally in Thailand, since the dedicated amino acid analyzers in various institutions in Thailand were often not functional. We have, therefore, sought to establish normal values of plasma amino acid levels in Thai children of various ages, using reversed-phase HPLC[13], since many HPLC instruments are available in Thailand, which may be adapted for analyzing amino acids.

Comparisons of amino acid levels for the five age groups in our study show few differences, so that normal values for different age groups are generally similar. However, noteworthy differences were found in hydroxyproline, which was significantly higher (at $p<0.01$ level) in group 1 (age 0 m-6 m). This is in agreement with the work of Ghisolfi et al[8], who could detect hydroxyproline in noticeable amounts up to the age of 6 m. A possible explanation for the elevated hydroxyproline levels in young infants of less than 6 m is that hydroxyproline is an important constituent of bones, teeth and connective tissue, which are being deposited after birth.

Statistical comparisons of the mean levels of the 20 free amino acids in plasma determined in this paper with data from other groups (Table 3) show that normal free plasma amino acid levels values in children vary considerably in different reports. These comparisons show that our data seem to be more similar to the data reported by Ghisolfi et al[8] since fewer amino acids differed by $p<0.05$, while the data of Sirichakwal et al[11] are similar to the results reported by Meites et al[7] using the same criterion. The similarity of our data to that of Ghisolfi et al[8] suggests that the HPLC technique used is an acceptable alternative, which can give

Table 3. Comparison of plasma amino acid levels (mean ± SD) in the present study with other reports by student *t*-test.

Amino acid nmol/ml	(A) Present study (1-3 yr) (n = 10) $\bar{X} \pm SD$	(B) Srichakwal (1-3 yr) (n = 10) $\bar{X} \pm SD$	(C) Ghisolfi (10 mo-3yr) (n = 36) $\bar{X} \pm SD$	(D) Meites (1-5 yr) (n=30) $\bar{X} \pm SD$	A& B	A&C	A&D	B&C	B&D	C&D
					Student t-test p-value					
Glutamic acid	144.3 ± 133.1	217.5 ± 72.0	64.5 ± 27.8	184 ± 106	0.148	0.089	0.343	0.000	0.359	0.000
Serine	105.6 ± 42.9	184.7 ± 46.6	134.2 ± 66.6	188 ± 52	0.000	0.208	0.000	0.030	0.862	0.000
Glycine	146.7 ± 47.5	302.6 ± 90.6	210.6 ± 54.6	260 ± 46	0.000	0.002	0.000	0.011	0.181	0.000
Taurine	80.6 ± 54.9	218.8 ± 54.3	95.8 ± 45.5	104 ± 61	0.000	0.378	0.291	0.000	0.000	0.547
Histidine	16.7 ± 11.9	87.1 ± 20.6	67.7 ± 14.1	103 ± 29	0.000	0.000	0.000	0.000	0.118	0.000
Citrulline	17.8 ± 12.8	27.9 ± 8.5	26.8 ± 10.8	27 ± 16	0.056	0.031	0.000	0.759	0.000	0.958
Threonine	59.5 ± 40.9	105.8 ± 28.5	89.0 ± 43.6	127 ± 43	0.009	0.062	0.000	0.258	0.157	0.001
Alanine	197.6 ± 84.8	448.3 ± 176.4	239.3 ± 76.4	492 ± 128	0.001	0.143	0.000	0.004	0.891	0.107
Arginine	56.3 ± 33.1	74.7 ± 50.5	59.7 ± 24.1	46 ± 31	0.349	0.715	0.376	0.384	0.116	0.047
Proline	113.1 ± 60.4	142.6 ± 72.1	157.3 ± 72.1	197 ± 74	0.159	0.084	0.002	0.570	0.003	0.032
α-Aminobutyrate	12.9 ± 6.9	17.5 ± 11.6	14.5 ± 5.81	20 ± 24	0.300	0.449	0.154	0.460	0.659	0.233
Tyrosine	44.7 ± 16.5	71.8 ± 26.5	48.6 ± 14.9	78 ± 27	0.013	0.484	0.001	0.022	0.533	0.000
Valine	158.3 ± 72.9	246.1 ± 87.1	183.7 ± 58.1	210 ± 82	0.025	0.253	0.056	0.054	0.243	0.147
Methionine	19.6 ± 9.2	28.1 ± 14.7	16.1 ± 6.7	21 ± 13	0.334	0.179	0.767	0.029	0.151	0.069
Isoleucine	40.5 ± 20.5	67.9 ± 16.0	51.1 ± 16.0	65 ± 29	0.004	0.089	0.019	0.005	0.691	0.024
Leucine	95.8 ± 47.6	145.8 ± 70.2	88.5 ± 25.1	142 ± 55	0.079	0.650	0.023	0.030	0.086	0.000
Phenylalanine	59.4 ± 13.4	76.3 ± 34.5	47.8 ± 10.3	63 ± 22	0.175	0.005	0.637	0.028	0.273	0.006
Tryptophan	13.0 ± 5.5	59.8 ± 24.0	28.4 ± 13.2	85 ± 40	0.000	0.000	0.000	0.007	0.069	0.000
Ornithine	31.7 ± 20.8	86.9 ± 31.7	44.6 ± 14.3	154 ± 53	0.000	0.029	0.000	0.002	0.000	0.000
Lysine	93.8 ± 60.7	169.1 ± 70.5	129.4 ± 38.3	174 ± 54	0.020	0.104	0.000	0.115	0.822	0.000

Table 4. Comparison of maximum and minimum levels of plasma amino acid levels between data in this paper with other reports.

Amino acid nmole/ml plasma	This paper (1 mo-12 yr) Range (n=57)	Srichakwal (1-19 yr) Range (n=63)	Shapira (1 mo-18 yr) Range	Behrman (>1 mo-16 yr) Range
Phosphoserine	0-140.4	-	1.0-30.0	0-12.00
Aspartic acid	0.9-20.9	18.1-84.2	0-24	5-59
Glutamic acid	25.4-525.2	55.1-373.2	5-150	0-210
α-Aminoadipic acid	0-14.1	-	0	-
Hydroxyproline	4.3-65.9	-	0-63	0-40
Phosphoethanolamine	0-11.8	-	0-69	-
Serine	44.4-275.4	147.6-309.5	69-187	90-226
Asparagine	29.3-144.9	-	21-112	28-246
Glycine	79.6-463.0	216.0-473.3	81-436	89-360
Glutamine	52.4-760.8	-	246-1,182	52-669
β-Alanine	0-23.7	-	0-7	-
Sarcosine	-	-	0-9	-
Taurine	8.4-278.8	78.3-331.5	10-170	22-192
Histidine	0-61.5	60.0-202.6	41-125	52-124
γ-Aminobutyric acid	<10	-	0	-
Citrulline	0.0-154.9	14.3-47.4	1-46	1-55
Threonine	12.1-403.5	78.2-219. 3	24-226	73-160
Alanine	76.4-575.9	270.8-767.4	143-547	142-484
β-Amino-isobutyric acid	<5	-	0	-
Carnosine	<5	-	0	-
Arginine	11.7-175.0	54.6-220.7	10-140	6-187
Proline	41.8-458.4	112.2-380.0	52-369	67-238
1-Methylhistidine	<5	-	0-44	0-27
Anserine	<5	-	0	-
3- Methylhistidine	<5	-	0-5	0-6
α-Amino-n-butyric acid	0-48.85	8.72-32.00	3-31	0-42
Tyrosine	17.91-406.69	23.76-139.78	22-115	29-86
Valine	61.35-367.68	142.74-441.88	64-321	110-271
Methionine	3.84-56.97	16.11-75.17	7-47	0-90
Cystathionine	-	-	0-5	-
Cysteine	-	-	5-84	0-106
Homocysteine	-	-	0-5	0-0
Isoleucine	15.2-106.5	42.8-145.0	22-107	34-85
Leucine	36.9-191.6	77.9-322.1	47-216	55-165
Hydroxylysine	<5	-	0-7	-
Phenylalanine	25.0-118.5	43.6-168.5	26-91	22-98
Tryptophan	2.3-40.8	16.7-110.0	0-79	24-79
Ornithine	5.2-120.1	43.1-214.1	10-163	15-143
Lysine	25.8-235.6	95.2-537.0	48-284	68-266
Ethanolamine	-	-	0-7	-

values similar to those obtained by ion-exchange chromatography. Moreover, the substantial differences between the two Thai reports and between the French and the American reports indicate that the plasma amino acids levels of Thai children and Western children do not appear to show any characteristic differences.

Comparisons of the maximum and minimum values of plasma free amino acid levels in the present work compared to the data of Sirichakwal et al[12] and to values reported in two textbooks [5,6] are shown in Table 4. These comparisons show that while, Shapira et al[5] listed values for 40 amino acids, we reported values for 35 amino acids, and Behrman et al[6] reported values for 29 amino acids, but Sirichakwal et al[12] only reported values for 21 amino acids. Typically, the failure to report values for an amino acid may be due to the low levels of the amino acid present in plasma or inadequate techniques for detection and quantitation.

Shapira et al[5] reported 4 amino acids present in low yield, sarcosine, ethanolamine, cystathionine and homocysteine, as well as one major amino acid, cysteine, not reported by the authors who were unable to report a value for cysteine, since in the chromatogram, this amino acid overlaps with a reagent peak. Because of this difficulty, the authors have developed an alternative method for analyzing sulfur amino acids, namely, free cysteine, homocysteine, and glutathione. The method, adapted from the procedure of Cornwell et al[23], involves prior treatment of the sample with tri-n-butyl phosphine in 10 per cent dimethyl formamide and reaction with 7-fluorobenzo-2-oxo-1, 3-diazole-4-sulphonamide, followed by isocratic separation of thiol derivatives by reversed phase HPLC and fluorescence detection; results will be reported elsewhere.

As noted earlier, data reported by Behrman et al[5] list fewer amino acids than that reported in the present paper, but the amino acids not reported were all low yield amino acids, namely α-amino-adipic acid, phosphoethanolamine, β-alanine, γ-aminobutyric acid, β-amino-isobutyric acid, carnosine, anserine, and hydroxylysine. On the other hand, Sirichakwal et al[12], who listed only 21 amino acids, not only lacked several low yield amino acids, but failed to report values for two major amino acids (asparagine and glutamine) and two moderate yield amino acids (phosphoserine, hydroxyproline). Since asparagine and glutamine are reported to be present in large amounts in the present work and that of Shapira et al[5] and Behrman et al[6] (Table 4), so their absence in the reports by Sirichakwal et al[12] are likely to be due to problems in separation and/or quantitation.

In conclusion, the present study has shown that plasma amino acid levels in children may be readily determined in Thailand by using reversed-phase HPLC coupled to pre-column derivatization. The method gives acceptable values when compared to other work done using dedicated amino acid analyzers, both locally and overseas. However, there were substantial variations between the values reported in different groups, so ideally, each laboratory interested in studying abnormalities in amino acid levels, for example due to inborn errors of metabolism, should develop its own data for normal values. From our data, the authors tend to prefer the use of 95 per cent confidence interval values, rather than using the maximum and minimum values, since the latter may have some unusually high or unusually low values. In terms of diagnosis of disease, it is better to include more possible cases for further follow-up by more detailed assays, such as enzyme levels in leukocytes or in cultured fibroblasts, rather than missing any cases due to high maximum or low minimum values. It is hoped that this work will encourage other groups in Thailand, interested in analyzing amino acids levels in physiological fluids, to consider setting up facilities for amino acid analysis by reverse-phase HPLC, since many institutions have HPLCs that can be adapted for this purpose. The values reported in the present study can be used as guidelines for normal values of plasma amino acids in Thai children, but ideally each group should establish their own normal values, due to the variations between different laboratories discussed here.

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REFERENCES

1. Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The Metabolic and Molecular Bases of Inherited Disease, Vol 1. New York: McGraw-Hill, 1995: 1239-77.
2. Scriver CR, Kaufman S, Eisensmith RC. The hyperphenylalaninemias. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The metabolic and molecular bases of inherited disease. 7th ed. New York, NY: McGraw-Hill, 1995: 1015-77.
3. Chuang DT, Shih VE. Disorders of branched chain amino acid and ketoacid metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The Metabolic and Molecular Bases of Inherited Disease, Vol 1. New York: McGraw-Hill, 1995: 1239-77.
4. Wasant P, Svasti J, Srisomsap C, Liammongkol S, Naylor E, Matsumoto I. Inherited Metabolic Disorder in Thailand - Siriraj Experience. Southeast Asian J Trop Med Public Health 1999; 30 (Suppl 2): 124-37.
5. Shapira E, Blitzer MG, Miller JB, Africk DK. Biochemical Genetics: A Laboratory Manual, New York: Oxford University Press, 1989a: 94-7.
6. Behrman RE, Kliegman RM, Jensen HB, (eds) Nelson Textbook of Pediatrics. Philadelphia: WB Saunders and Co., 2000: 2189.
7. Meites S, ed. Pediatric Clinical Chemistry, 3rd ed., Washington DC: American Association for Clinical Chemistry, 1988.
8. Ghisolfi J, Augier D, Regnier C, Dalous A. Etude des variations physiologiques en fonction de l'age du taux des acides amines libres plasmatiques chez l'enfant normal. Arch Franc Ped 1973; 30: 951-7.
9. Armstrong MD, Stave U. A study of plasma free amino acid levels. II. Normal values for children and adults. Metabolism 1973; 22: 561-9.
10. Wu PYK, Edwards N, Storm MC. Plasma amino acid pattern in normal term breast-fed infants. J Pediat 1986; 109: 347-9.
11. Suvanapha R, Tungsanga K, Laorpatanaskul S, Sitprija V, Suwan S. Plasma amino acid patterns in normal Thais and in patients with chronic renal failure. J Med Assoc Thai 1991; 74: 211-6.
12. Sirichakwal P, Feungpean B, Tontisirin K. Plasma free amino acid contents in healthy Thai subjects. J Med Assoc Thai 1999; 82 (Suppl 1): S129-36.
13. Cohen SA, Meys M, Tarvin TL. The Pico-Tag Method. A manual of advanced techniques for amino acid analysis. Milford, USA: Waters Chromatography Division, Millipore Corporation, 1989.
14. Matteson KJ. HPLC analysis of amino acids in inborn errors of metabolism. Southeast Asian J Trop Med Public Health 1995; 26 (Suppl 1): 120-2.
15. Spackman DH, Stein WH, Moore S. Automatic recording apparatus for use in the chromatography of amino acids. Anal Chem 1958; 30: 1190-206.
16. Hamilton PB. Ion-exchange chromatography of amino acids. A single column, high resolving, fully automatic procedure. Anal Chem 1963; 35: 2055-64.
17. Hamilton PB. Micro and submicro determinations of amino acids by ion-exchange chromatography. Methods Enzymol 1967; 11: 15-27.
18. Slocum RH, Cummings JG. Amino acid analysis of physiological samples. In Techniques in Diagnostic Human Biochemical Genetics: A laboratory manual. New York: Wiley-Liss Inc., 1991: 87-127.
19. Kaiser FE, Gehrke CW, Zumwalt RW, Kuo KC. Amino acid analysis. Hydrolysis, ion-exchange cleanup, derivatization and quantitation by gas-liquid chromatography. J Chromatogr 1974; 94: 113-33.
20. Bayer E, Grom E, Kaltenegger B, Uhmann R. Separation of amino acids by high performance liquid chromatography. Anal Chem 1976; 48: 1106-9.
21. Heinrikson RL, Meredith SC. Amino acid analysis by reverse-phase high performance liquid chromatography: Pre-column derivatization with phenylisothiocyanate. Anal Biochem 1984; 136: 65-74.
22. Lin J-K, Wang C-H. Determination of urinary amino acids by liquid chromatography with "dabsyl chloride". Clin Chem 1980; 26: 579-83.
23. Cornwell PE, Morgan SL, Vaughn WH. Modification of a high-performance liquid chromatographic method for assay of homocysteine in human plasma. J Chromatogr 1993; 617: 136-9.

ระดับของกรดอะมิโนอิสระในพลาสม่าของเด็กไทย

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การวิเคราะห์ปริมาณกรดอะมิโนอิสระในพลาสม่ามีความสำคัญในการวินิจฉัยโรคพันธุกรรมที่เกี่ยวกับเมตาบอลิสม (Inborn errors of metabolism) ปกติการวิเคราะห์นี้ จะใช้เครื่อง amino acid analyser แต่ในประเทศไทยมีเครื่องที่ทำงานได้ในจำนวนน้อย การวิจัยนี้จึงได้วิเคราะห์ปริมาณกรดอะมิโนอิสระในเด็กปกติโดยใช้เครื่อง HPLC แทน และได้รายงานผลเป็นค่า mean ± SD และ 95% Confidence Interval สำหรับกลุ่มอายุ 0–6 เดือน, 6–12 เดือน, 1–3 ปี, 3–6 ปี, 6–12 ปี ในกลุ่มอายุ 0–6 เดือน พบว่าโดยทั่วไประดับกรดอะมิโนอิสระไม่มีความแตกต่างกันระหว่างกลุ่ม ($p \geq 0.01$) ยกเว้น hydroxy-proline ซึ่งมีค่าสูงกว่ากลุ่มอายุอื่น

วิธีการ HPLC ที่ใช้นี้สามารถแยกและวิเคราะห์หาปริมาณของกรดอะมิโนอิสระได้ถึง 35 ชนิด มากกว่าที่เคยรายงานมาก่อนในประเทศไทย และค่า mean ± SD นั้น คล้ายกับผลที่เคยรายงานในต่างประเทศ จึงน่าจะได้พิจารณาใช้เครื่อง HPLC เพื่อวิเคราะห์ระดับของกรดอะมิโนอิสระในพลาสม่าในประเทศไทย แต่ทั้งนี้ผลที่ได้จากห้องปฏิบัติการแต่ละแห่งอาจมีความแตกต่างกันมากพอสมควร ดังนั้นห้องปฏิบัติการแต่ละแห่งควรหาระดับของกรดอะมิโนอิสระในเด็กปกติเพื่อใช้เปรียบเทียบกับค่าที่ได้ในเด็กป่วย

คำสำคัญ : กรดอะมิโน, โรคพันธุกรรม, พลาสม่า

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