International Journal of Health Sciences and Research
Vol.13; Issue: 11; November 2023
Website: www.ijhsr.org
Original Research Article *ISSN: 2249-9571*

Plasma Amino Acids Profile: Established Reference Range in Indian Children

Komal Uppal[1], Richa Nijhawan[2], Sunil Kumar Polipalli[3], Sanjeev Kumar Pandey[4], Avinash Lomash[5], Somesh Kumar[6], Ankur Jindal[7], Seema Kapoor[8]

[1,2,8]Paediatrician, [3,5,6,7]Scientist, [4]Assistant Professor,
[1,2,3,5,6,7,8]Division of Genetics and Metabolism, Department of Pediatrics, Maulana Azad Medical College (Delhi University), Delhi 110002
[4]Hind Institute of Medical Sciences, Uttar Pradesh
[5]Medanta - The Medicity, CH Baktawar Singh Rd, Medicity, Islampur Colony, Sector 38, Gurugram, Haryana 122001

Corresponding Author: Sunil Kumar Polipalli

DOI: https://doi.org/10.52403/ijhsr.20231136

ABSTRACT

Reference values were determined for 17 plasma free amino acids from measurements done in 248 healthy children ranging from 0 to 12 years of age. The method used is based on precolumn derivatization of primary amino acids with o-phthalaldehyde (OPA) and 9-fluorenylmethy[l] chloroformate (FMOC) for secondary amino acids, separation of the derivatives by reversed-phase chromatography, and quantification by fluorescence detection. We propose to present the age-specific distribution of plasma amino acid concentrations. Complete separation was achieved within 18 min. Total analysis time, including derivatization, chromatography, and reequilibration of the column, was 25 min. The assay was linear from 0 to 518 umol/L for all amino acids. Recovery of amino acids added to plasma samples was 92-104%. Within-run precision (CV) was 0.21-6.41% and between- run precision was 3.34-15.75%. This way of presenting amino acid concentrations may facilitate the follow-up of patients with inborn errors of amino acid metabolism.

Keywords: Free Amino Acids, Reversed-phase chromatography.

INTRODUCTION

Amino acid analysis by reversed-phase chromatography is a procedure extensively used for diagnosis and follow-up of inborn errors of metabolism. Reversed-phase chromatography provides both highly consistent and sensitive results. Various advanced techniques like gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS/MS) to do amino acid analysis have higher costs, so, reversed-phase chromatography is still an important tool for the amino acid analysis in most of the laboratories in developing countries. Normative values for plasma amino acid concentrations have been reported in adults [1– 4], in infants ages 0–2 months [5] and 1–5 months [6], and in older children ages 6–18 years [1, 7, 8]. Mean concentrations with individual SDs for the selected population to confer these data are used [1, 2, 4, 6–8]. As, there can be considerable discrepancies between the reference range from different population and laboratories from different countries, so, each country and laboratory should set its own reference range for amino acids. By comparing the patient value with population specific reference range, we can detect amino

acidopathies early which can often help in early management measures in the form of dietary modifications and vitamin supplementation, prevent multiorgan damage in the baby. However, no Indian data describe the age-specific distribution of amino acid concentrations from infancy to childhood. In the present paper, we propose a method of analyzing results that takes advantage of all amino acid values. The distribution of amino acid concentrations is presented from birth to 12 years of age. Thus, the amino acid values of a patient with a particular metabolic disease can be easily compared from infancy to adulthood with those of the control population of the appropriate age.

MATERIALS & METHODS

Sodium acetate trihydrate, sodium hydroxide, boric acid, 5- sulfosalicyclic, acetonitrile (LC grade), and methanol (LC grade) were obtained from Merck (Amsterdam, The Netherlands). OPA and FMOC reagent was obtained from Agilent Alto, CA. Tetrahydrofuran was supplied by J. T. Baker (Deventer, The Netherlands), Triethylamine was obtained from Aldrich (Brussels, Belgium) Borate buffer was prepared by adjusting 0.4 N boric acid to pH 10.2 with NaOH. Amino acid standard mixtures at the concentration of 25 pmol /µL were from Agilent. The internal standard l-norvaline was procured from Sigma-Aldrich Company. Analysis was performed by Agilent 1100 liquid chromatography using Hypersil AA-ODS 5um, 2.1 x 200mm column.

Preparing mobile phases and standards

Mobile phase A:

1. Weigh 1.36 ± 0.025 g of sodium acetate tri-hydrate and transfer it into a 800 ml glass beaker.
2. Add 500 ml of purified water and stir until all crystals are completely dissolved.
3. Add 90 µl of triethylamine (TEA) and mix.
4. Adjust the pH to 7.20 ± 0.05 by adding a few drops of 1–2 % acetic acid.
5. Add 1.5 ml of tetrahydrofuran (THF) and mix.

Mobile phase B:

1. Weigh 1.36 g ± 0.025 g of sodium acetate trihydrate and transfer it into a 200 ml glass beaker.
2. Add 100 ml of purified water and stir until all crystals are dissolved.
3. Adjust pH to 7.20 ± 0.05 by adding a few drops of 1–2 % acetic acid.
4. Add this solution into a mixture of 200 ml acetonitrile and 200 ml methanol and mix.

Amino acid standard solutions

Amino acid standard samples were prepared by mixing 95 µL of the 25 pmol/µL amino acid standard mixture with 5 µL of 10 mM norvaline and analyzed directly by RP-HPLC, within 24 h from preparation. Solutions for linearity study were prepared in duplicate by diluting the 1 nmol/µL amino acid standard solution, and contained 20, 50, 130, 250, or 500 pmol/µL of amino acid standard mixture together with 0.5 mM norvaline.

Subjects

To determine reference values for amino acids, we analyzed plasma samples from 248 healthy children (ages 0-12 years, 168 boys and 80 girls). Blood samples were obtained after an overnight fast. Informed consent has been taken from each subject.

Collection and Processing of Samples

Tubes containing 20 mg of deproteinizing reagent SSA were prepared as follows: A solution of 200 g/L SSA in absolute ethanol was divided into 100ul aliquots in 1.0-mL tubes. After evaporation of the ethanol in an oven (overnight at 50°C), the tubes were capped and stored at room temperature. These SSA tubes contain sufficient SSA to deproteinize 0.5 mL of plasma. Venous

blood was collected in EDTA vials. After centrifugation (2000g for 10 min at 4°C), 0.5 mL of plasma was pipetted into a SSA tube. The tubes were vigorously mixed on a vortex-type mixer, snap-frozen in liquid nitrogen, and then stored at -70°C.On the day of analysis the tubes were thawed at room temperature and centrifuged (15 min at 3000g and 4°C) to remove precipitated protein. The clear supernatants were transferred to polypropylene vials and stored in a refrigerated (4°C) condition. All the factors which can be responsible for variation in the result analysis have been standardized and taken care of.

Chromatographic conditions

Column: 200 x 2.1 mm AA column and guard column
Mobile Phase: A= 20mMol NaCl + 0.018 % TEA adjusted to pH 7.2
B = 20 % of mMol NaCl adjusted to pH 7.2 with 1-2 % acetic acid + 40 % CAN and 40 % MeOH
Flow Rate: 0.45 ml/min
Gradient: start with 100%A, at 17 min 60% B, at 18min 100% B, at 18.1 min flow 0.45, at 18.5 min flow 0.8, at 23.9 min flow 0.8, at 24min 100%
B and flow 0.45, at 25 min 0% B.
Oven Temp: 40 ^{0}C
Post Time: 5min

Injector program:

Take 100 µl borate buffer
Take 20µl OPA reagent
Take 20µl sample
Mix properly
Take 20 µl FMOC
Mix Properly
Inject 20 µl

FLD Setting:

Excitation = 340 nm Emission = 450nm PMT gain 12
at 14.5 min
Excitation = 266 nm Emission = 305 nm PTM Gain 11

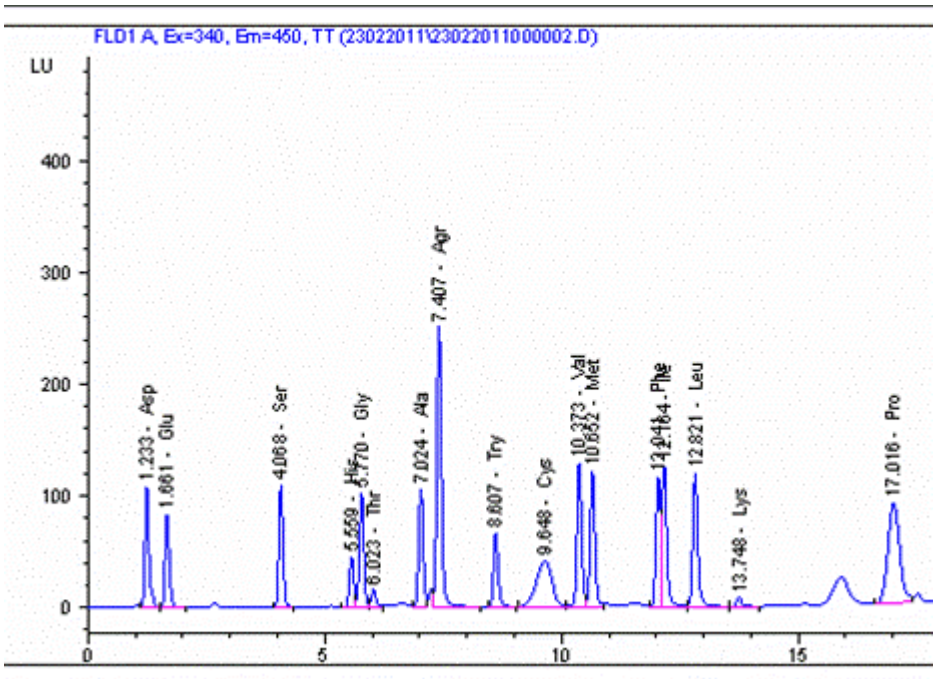

Fig 1: Analysis of 25 pmol amino acids standard with florescence detection

RESULT

Fig. 1 shows the chromatographic separation of an amino acid standard and a plasma sample. Eluent composition and gradient shape were optimized to obtain baseline resolution of all amino acids. Some critical pairs of amino acids are difficult to separate. To obtain baseline separation of glycine and threonine we found it essential to include a small percentage of

tetrahydrofuran in mobile phase A. Separation of tryptophan and phenylalanine was accomplished by optimizing the ratio of methanol and acetonitrile in mobile phase B. Inclusion of triethylanilne in mobile phase A allowed separation of aspartic acid from SSA used to deproteinize the plasma samples. The elution position of histidine and arginine relative to the other amino acids can be shifted by adjusting the ionic strength of the mobile phase buffer. After determining the optimal composition of the eluents, fine-tuning of the separation was performed by modifying the gradient shape. By using a segmented gradient, resolution was improved with a concurrent reduction of analysis time. There was a linear relation between peak response (ratio of areas of amino acid and internal standard) and concentration from 0 to 518 µmoI/L for all amino acids (correlation coefficients all >0.999). Within-run precision was determined by replicate analysis of an amino acid standard solution (n = 25) and a plasma sample (n = 248). For the standard solution the CV was 1.0-1.8%. For the plasma sample the CV was 1.8-4.9% with the exception of aspartic acid (6.4%). For quality-control purposes a plasma pool was stored at – 70^0C in small aliquots; this sample was analyzed in each run. Amino acid concentrations determined in plasma of 248 healthy children are summarized in Table 1.

Table1. Plasma amino acid values (µmol/L) from control children of selected age.

	0-3 months	4-12 months	1-5 years	6-12 years
Aspartic acid	0-9.9	1.65-12.3	2.0-11.4	3.0-14
Glutamic acid	30-151	46-188	32-167	11-138
Serine	43-150	83-205	72-165	49-135
Histidine	13-57	12.7-87.6	13-70	15-50
Glycine	116-401	146-237	120-345	100-275
Threonine	20-140	36-193	43.3-150	25-88
Alanine	123-518	226-396	119-388	144-370
Arginine	23-152.2	31.7-157	7-151	8.8-109
Tyrosine	22-100	16-112	19-85	7.7-105
Cystine	107-300	96.6-345	120-484	100-394
Valine	57-282	71-282	98-251	47-192
Methionine	15-77.3	10-48	12.2-48.3	9-48.9
Phenylalanine	23-131	19- 131	34-83	22-131
Isoleucine	4.4- 94.1	9-93	24.5-109	11.3-103
Leucine	36-192	39-190	47-190	15.3-188
Lysine	21-148	43-135	55-140	21-148
Proline	63-201	52-212	130-210	63-151

DISCUSSION

The objective of this study was to develop a reference range for the analysis of plasma amino acids in children of Indian subcontinent. Precolumn derivatization with OPA was used because the derivatization can be easily automated. To minimize problems due to oxidation of the reagent [9], we kept it in multiple capped vials. Each vial was used for the derivatization of ten consecutive samples. A potential source of analytical variation may be the instability of the isoindole derivatives formed by reaction with OPA[10]. Assuming a total amino acid concentration of appox.3 mmol/L for plasma, we used OPA at a 10-fold molar excess over total amino acids. In initial experiments with higher OPA concentrations, we observed that some amino acid derivatives (i.e., glycine, histidine, and taurine) degraded, and some additional unidentified peaks probably representing degradation products appeared in the chromatograms. This is in accordance with reports that excess OPA accelerates degradation of isoindole derivatives [10]. On-column degradation of the derivatives was minimized by using a short column in combination with a high flow rate, resulting in an analysis time of only 25 min. To obtain sufficient resolution, we used a column containing 5 nm particles. An additional advantage of these small-diameter particles is that resolution is only marginally decreased at high flow rates, due to a more efficient mass transfer. To obtain

reliable values for plasma amino acids, we paid special attention to sample pretreatment. After collection of blood, plasma was immediately separated from blood cells by centrifugation, and plasma proteins were then precipitated by addition of SSA. In a comparison of various deproteinizing agents, Qureshi and Qureshi showed SSA to be superior to other agents [12]. We used special tubes containing solid SSA to avoid errors due to sample dilution. After mixing within the tubes, the samples were quickly frozen in liquid nitrogen and then stored at -70C. In our experience, samples prepared in this way can be stored for at least 1 year without change in amino acid concentrations. This method is characterized by high precision and reproducibility for all amino acids evaluated. With one exception, the recoveries of amino acids added to plasma were close to 100%. The lower recovery of tryptophan (89%) probably reflects the fact that this amino acid is tightly bound to albumin [15]. Plasma amino acid concentrations of healthy subjects determined by the present method (Table 1) are in good agreement with values obtained by other investigators [11-14,17,18]. Although we are primarily interested in plasma amino acids, the method can also be applied to the determination of amino acids in cerebrospinal fluid. The method is characterized by speed of analysis and minimal manual sample handling. With proper care the column can be used for at least 400 analyses. If the separation efficiency decreases, column performance can be restored by regenerating the column with water, methanol and acetonitrile.

CONCLUSION

In conclusion, HPLC method is a simple, rapid, and reliable method for the quantitative measurement of plasma amino acids. The established reference range of plasma amino acids for younger Indian children will guide us in the diagnosis and management of diseases that effect amino acid metabolism and Inherited metabolic disorders.

Conclude your research paper here.

Declaration by Authors

Ethical Approval: Approved

Acknowledgement: Gratitude to the Division of Genetics MAMC and Participants.

Source of Funding: None

Conflict of Interest: The authors declare no conflict of interest.

REFERENCES

1. Armstrong MD, Stave U. A study of plasma free amino acid levels. II. Normal values for children and adults. Metabolism 1973; 22: 561–9.
2. Perry TL, Hansen S. Technical pitfalls leading to errors in the quantitation of plasma amino acids. Clin Chim Acta 1969; 25: 53–8.
3. Caballero B, Gleason RE, Wurtman RJ. Plasma amino acid concentration in healthy elderly men and women. Am J Clin Nutr 1991; 53:1249–52.
4. Moller SE. Quantification of physiological amino acids by gradient ion-exchange high-performance liquid chromatography. J Chromatogr 1993; 613:223–30.
5. Janas LM, Picciano MF, Hatch TF. Indices of protein metabolism in term infants fed human milk, whey-predominant formula, or cow's milk formula. Pediatrics1985; 75:775–84.
6. Lindblad BS, Alfven G, Zetterstrom R. Plasma free amino acid concentrations of breast-fed infants. Acta Paediatr Scand 1978; 67:659–63.
7. Gregory DM, Sovetts D, Clow CL et at. Plasma free amino acid values in normal children and adolescents. Metabolism 1986; 35:967–9.
8. Walker V, Mills GA. Quantitative methods for amino acid analysis in biological fluids. Ann Clin Biochem 1995; 32:28–57.
9. May ME, Brown LL. Instability of orthophthalaldehyde reagent for amino acid analysis. Anal Biochem 1989; 181:135-9.
10. Alvarez-Coque MCG, Hernandez MJM, Camafias RWV et al. Formation and instability of o-phthalaldehyde derivatives

of amino acids. Anal Biochem 1989; 178:1-7.
11. Fermo I, De Vecchi E, Diomede L et al. Serum amino acid analysis with pre-column derivatization: comparison of the o-phthaldialdehyde and N, N-diethyl-2,4-dinitro-5-fluoroaniline methods. J Chromatogr 1990; 534:23-35.
12. Qureshi GA, Qureshi AR. Determination of free amino acids in biological samples: problems of quantitation. J Chromatogr1989; 491:281-9.
13. Blundeli G, Brydon WG. High performance liquid chromatography of plasma amino acids using orthophthalaldehyde denivatisation. Clin Chim Acta 1987; 170:79-84.
14. Godel H, Graser T, Foldi P et al. Measurement of free amino acids in human biological fluids by high-performance liquid chromatography. J Chromatogr 1984; 297:49-61.
15. Uhe AM, Coffier GR, McLennan BA et al. Quantitation of tryptophan and other plasma amino acids by automated precolumn o-phthaldialdehyde derivatization high-performance liquid chromatography: improved sample preparation. J Chromatogr 1991; 564:81-91.
16. Van EiJk HMH, Vanider Hejden MAN, Van Berlo CLH et al. Fully automated liquid-chromatographic determination of amino acids. Clin Chem 1988; 34:2510-3.
17. Ziegler F, La Boucher J, Coudray-Lucas C et al. Plasma amino-acid determinations by reversed-phase HPLC: improvement of the orthophthalaldehyde method and comparison with ion exchange chromatography. J Autom Chem1992; 14:145-9.

How to cite this article: Komal Uppal, Richa Nijhawan, Sunil Kumar Polipalli, Sanjeev Kumar Pandey, Avinash Lomash, Somesh Kumar et.al. Plasma amino acids profile: established reference range in Indian children. *Int J Health Sci Res.* 2023; 13(11):304-309. DOI: *https://doi.org/10.52403/ijhsr.20231136*

******