# Biological Reference Interval of Amino Acids in the Dried Blood Spot of Term Neonates of South Karnataka Measured by High Performance Liquid Chromatography

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**Arjun Asok[1], Varashree Bolar Suryakanth[1], Leslie Edward Simon Lewis[2] and Prasanth C.B. Warrier[3]**

## Abstract

**Introduction:** The advent of newborn screening across India has led to an increase in the early diagnosis of inborn errors of metabolisms (IEMs). Aminoacidopathies, the group of inherited disorders of amino acid metabolisms are of particular important because of the ease of early diagnosis and the availability of effective treatment options. Unfortunately, the biological reference intervals for amino acids vary widely between different ethnic groups and geographical locations, thereby necessitating the need to establish a population specific reference interval for optimal diagnosis.

**Aims and objectives:** Establishment of the biological reference interval for all amino acids in the Dried Blood Spots (DBS) of term neonates belonging to coastal Karnataka using High Performance Liquid Chromatography.

**Methods:** Heel prick blood samples were collected from 175 healthy, term neonates on a filter paper. After safe transport to the laboratory, the amino acids were extracted using an appropriate solution, derivatized, and analyzed using high performance liquid chromatography. Mean, standard deviation, and 95% confidence interval of the mean were used to establish the reference interval. Students T-test was used to compare the differences in amino acids levels among different groups.

**Conclusions:** The biological reference intervals obtained in this study was found to have significant variations from studies conducted elsewhere in the world. This puts into perspective the need to establish a population specific reference interval for these parameters to avoid potential misdiagnosis. This reference interval may also be adopted by other labs catering to new-born screening in the same geographical area.

## Keywords

Dried blood spot, Aminoacidopathies, Biological reference interval, HPLC

## Introduction

With the increasing popularity of new-born screening worldwide, there has been a dramatic increase in both the detection as well as the early treatment of metabolic disorders. Among the most common metabolic disorders are a group of disorders called Aminoacidopathies, which present with varying degrees of defects in the metabolism of amino acids. Establishment of Aminoacidopathies is done by quantifying the amount of amino acids in the neonatal circulation. To make a diagnosis of aminoacidopathy, it is important to compare the amino acid levels in circulation to an established reference interval.

In the past, inborn errors of metabolisms were noticed in an individual only after they start showing overt symptoms, ranging from mild to severe. Even then, the diagnosis used to be non-specific in many cases since most laboratories do not do the necessary investigations routinely. This was further

[1]Manipal Academy of Higher Education, Kasturba Medical College, Department of Biochemistry, Manipal, Karnataka, India.
[2]Manipal Academy of Higher Education, Kasturba Medical College, Department of Paediatrics, Manipal, Karnataka, India.
[3]Sree Keralavarma College, Department of Statistics, Thrissur, India.

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**Corresponding Author:**
Varashree Bolar Suryakanth, E-mail: varashree.bs@manipal.edu

compounded by the difficulty in storing and transporting the appropriate samples (generally whole blood, plasma, or urine) to these laboratories. The advent of Dried Blood Spot (DBS) proved to be a major breakthrough with its unique advantages of easier sample collection, storage, transport, and the comparative stability of the measured analytes [1]. Owing to these advantages, new-born screening has become a standard of care practice in many countries across the world, both as a regional health policy and as a national health policy [2].

### *Newborn Screening*

New-born screening (NBS) is a series of examinations and investigations done in a new-born by a clinician or a laboratory scientist to screen the new-born for early identification of potentially devastating disorders, if any. The NBS programme is multifaceted and comprises of raising awareness among the parents and healthcare providers, collection and analysis of samples, treatment planning, and follow up for positive cases [3]. It includes routine clinical examinations including (but not limited to) checking the new-born for any anatomical defects (including cleft lip and palate), the patency of the anal opening and the external genitalia, a screening test to check for hearing and a blood test for screening of any metabolic perturbances. The impact of new-born screening on neonatal health has been so immense that it has been included in the list of ten great public achievements between 2001 to 2010 by the Centre for Disease Control and Prevention [4]. This article focuses on the laboratory aspects of the new-born screening.

### *Newborn Screening in India*

The first incidence of new-born screening in India was done in the year 1980 when a cohort of 1.25 lakh new-borns were screened for multiple disorders, particularly aminoacidopathies using thin-layer chromatography (qualitative analysis). The results of the study showed that aminoacidopathies were prevalent in the studied population at a much higher rate (particularly hyperphenylalaninemia, tyrosinemia, and maple syrup urine disease) than once thought and were an important cause of mental retardation and developmental delays [5]. This was followed by a multi-centric study conducted by the India Council of Medical Research (ICMR) across four cities and concluded that the aetiology of 5% of mental retardation in the screened population were attributed to aminoacidopathies [6]. In 2010, a study was conducted on 3,500 symptomatic patients, proving once again that the cause for symptoms in nearly 4% of these patients were metabolic disorders, the majority (54%) being aminoacidopathies [7].

Various studies have been done on establishment of reference intervals of amino acids in neonates in different parts of the world. Unfortunately, adhering to these established reference intervals may result in erroneous diagnosis since various studies have proved that the amino acid concentration in neonates changes dramatically depending on a variety of factors including (but not limited to) diet, gender, maternal age, maternal diet, and geographical location [8–10]. Hence, it becomes a necessity for laboratories doing new-born screening for aminoacidopathies to establish their own population-specific reference intervals. To the best knowledge of the authors, no study has been conducted in Southern India to try and establish a biological reference interval for amino acids in the dried blood spot of new-borns. As such, our laboratory, which screens up to 500 new-borns per month too has been using a biological reference interval established for a different population to what we are catering to. Even though Liquid chromatography – Mass Spectrometry (LC-TMS) remains the gold-standard for quantification of amino acids [11], infrastructural limitations in most of the laboratories results in the amino acid quantification using High Performance Liquid Chromatography (HPLC).

The aim of this study was to address this gap by establishing a population-specific reference interval for amino acids in the dried blood of new-borns belonging to Coastal Karnataka using HPLC. The study also explores the potential effect of external factors such as the mother's diet, time of sample collection, and type of delivery on the biological reference intervals of amino acids in dried blood spots.

## Materials and Methods

The study was conducted at the New-born screening laboratory, Centre for Excellence in Inborn Errors of Metabolism, Department of Biochemistry, Kasturba Medical College, Manipal. The archived samples that were sent for routine new-born screening to the laboratory were retrieved and used to quantify the amino acids present using HPLC.

### *Sample Size*

To establish a biological reference interval for the analytes of interest, an estimated sample size of 175 dried blood spot samples with a 2-sided 95% confidence interval with a distance from the mean for a precision of 15% and a standard deviation of 100 was considered necessary.

### *Sample Selection Criteria*

The collection of samples were done after the study had been cleared by the Institute Ethics Committee. The study, designed as a retrospective cross-sectional study was conducted between April and August 2022. As a routine laboratory procedure, DBS samples are sent to our new-born screening laboratory for the screening of seven parameters (Phenylketonuria (PKU), Maple Syrup Urine Disease (MSUD), Galactosemia, Glucose 6 phosphate dehydrogenase (G6PD) deficiency, congenital hypothyroidism, Biotidinase deficiency, and congenital adrenal hyperplasia. Normal samples are archived for a duration of three months whereas abnormal samples are archived for a duration of two years.

For this study, the normal samples were retrieved from the archive, after they had been processed for NBS. Care was taken to ensure that only those samples collected between 24 to 72 hours after delivery were analysed (DBS is collected from neonates between 24-72 hours after birth as a standard of care in the present study centre). Moreover, only those samples were selected which fit into the inclusion and exclusion criteria for the study (Table 1).

## *Sample Processing*

Quantification of amino acids using HPLC involved punching out discs of a suitable size using a DBS puncher, extracting the analytes of interest (amino acids in this case) from the DBS using 80% methanol and β-mercaptoethanol, preparing the sample, derivatizing it for fluorescent detection using o-phthaldaldehyde, and finally, quantifying based on the area under the curve. The volume of blood within the punched-out disc is important while quantifying the amino acids in blood. The amount of blood in a fixed diameter of DBS is dependent on a variety of factors like the completeness of sample collection, the hematocrit, and the exact method of sample collection. For the purpose of quantification, it was assumed that a 3 mm disc contained on an average 4 μL of blood [12,13].

For analysis of the samples, the reagents used were borate buffer (pH: 9), start buffer (pH: 6.4), and o-phthaldaldehyde (OPA) in 100% ethanol as a derivatizing agent.

Both the standards and the samples were treated with the OPA mix before injecting into the HPLC machine. A mixture of 25μL OPA mix, 100μL of supernatant, and 875μL of the start buffer would be filtered and then, injected into the HPLC machine. The program details for the HPLC run is given in Table 2.

The amino acids standards were run in different concentrations ranging from 1000 μmol/L to 10 μmol/L and the calibration curve was plotted. Individual amino acid peaks were identified by comparing their retention times (RT) with that of the amino acid standard mix. The amino acids from patient samples were extracted using methanol and β-mercaptoethanol [14]. The chromatograms from patient samples were overlaid with the chromatograms from the standard, area under the curve comparted and concentration quantified.

The retention times of each amino acids are shown below (Table 3 and Figure 1).

## *Statistical Method Used*

Mean, standard deviation, and 95% confidence interval of the mean were calculated using Microsoft Excel.

**Table 1.** Inclusion and Exclusion Criteria for DBS sample collection.

| Inclusion criteria | Exclusion criteria |
|---|---|
| Hospital born term neonates (vaginal delivery or Caesarean section) of either gender | Pre-term neonates |
| Birth weight beyond 2.5 kilograms | Low birthweight neonates |
| Exclusively breast fed | DBS collected after 72 hours of birth |
| No obvious phenotypical defects | On formula/supplement feeds |
| | Any phenotypical defects |
| | Mothers with gestational diabetes |
| Maternal age between 20 and 35 years | Mothers with pre-eclampsia |
| | Mothers with a bad obstetric history (more than 2 spontaneous abortions) |
| | Multiple pregnancies |

**Table 2.** Run details for quantification of DBS amino acids.

| Parameter | Details of importance |
|---|---|
| Detector | Fluorescence detector (RF-20A) |
| Excitation wavelength | 350 nm |
| Emission wavelength | 450 nm |
| Stationary phase | C18 Column (Purosper), 250mm (L), 5μM particle size |
| Mobile phase | Acetate buffer + Methanol |
| Total Run Time | 45 minutes |
| Software used | LC Solution/LC Browser |

**Table 3.** The retention time for amino acids in the current program.

| Identification | Amino acid | Retention time (minutes) |
|---|---|---|
| a | Aspartate | 9.74 |
| b | Glutamate | 12.38 |
| c | Asparagine | 16.82 |
| d | Serine | 17.98 |
| e | Glutamine | 18.6 |
| f | Glycine | 20.7 |
| g | Arginine | 21.4 |
| h | Threonine | 22.2 |
| i | Histidine | 23.1 |
| j | Tryptophan | 26.4 |
| k | Tyrosine | 27.2 |
| l | Alanine | 33.4 |
| m | Methionine | 34.2 |
| n | Valine | 35.4 |
| o | Phenylalanine | 36.2 |
| p | Isoleucine | 37.1 |
| q | Leucine | 39.1 |
| r | Lysine | 41.1 |

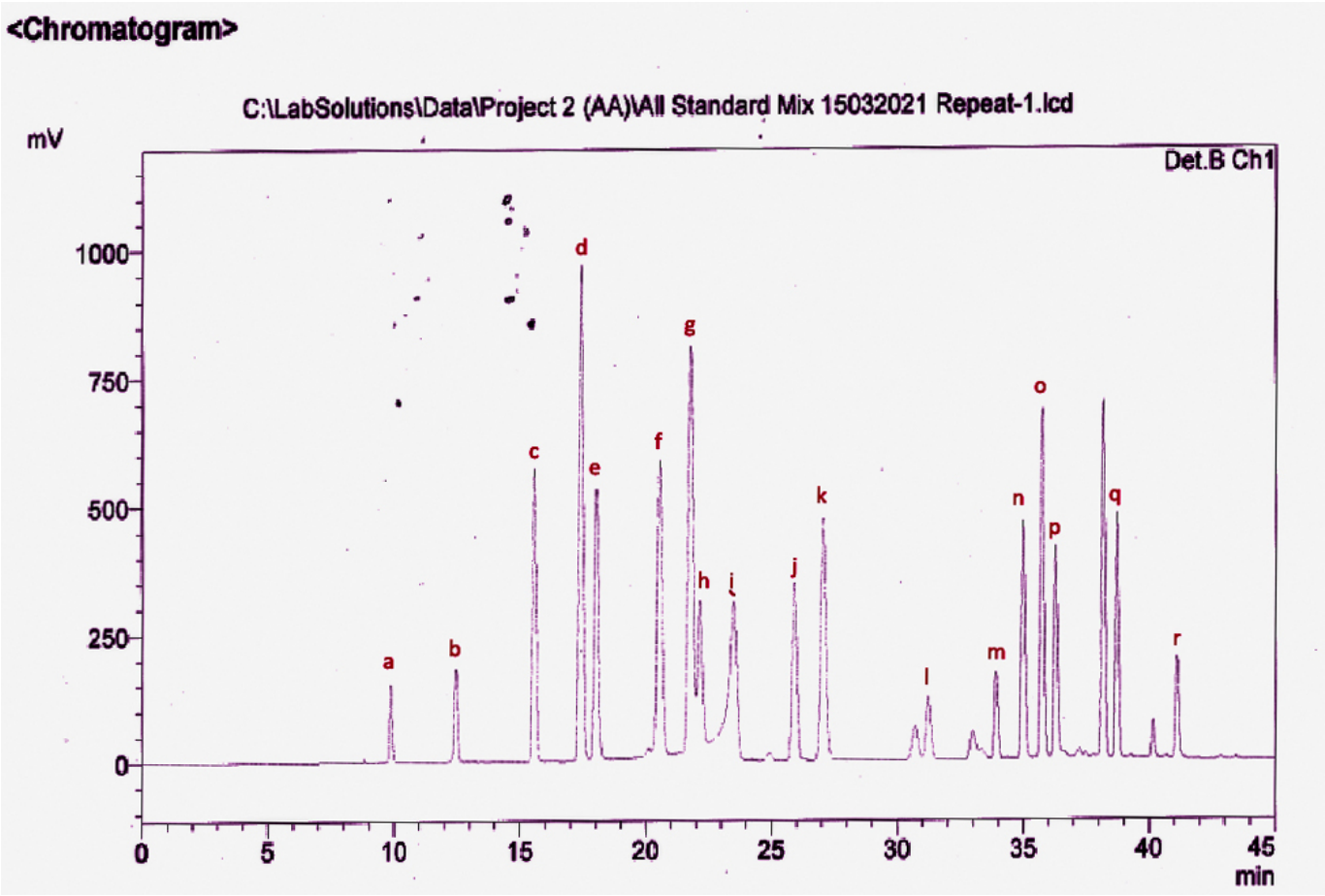


**Figure 1.** Retention times for all amino acids for the current test conditions.

T-test was used to compare the differences in the amino acid levels among the different groups (ie between vegetarian and mixed diet, and between normal vaginal delivery and caesarean section). Values were considered to be significant if the p-value was found to be less than 0.05.

## Results

A total of 175 samples were analyzed and the amino acid levels quantified. Out of the total, 109 were males and 66 were females. 58 of these samples were obtained from neonates born through cesarean section (either emergency or elective) and 117 were born through normal vaginal delivery. Descriptive statistics obtained from all the amino acids in the patients' samples are compiled (Table 4).

No amino acid concentrations showed any statistically significant difference based on the mother's diet except methionine, which showed an increase in those newborns whose mothers consumed a mixed diet ($p = 0.040$).

No amino acid concentrations showed any statistically significant difference based on the mode of delivery except tryptophan, which showed an increase in those newborns whose were born through normal vaginal delivery ($p = 0.041$).

## Discussion

Considering the vast differences in the major studies on the biological reference intervals of amino acids in neonates and the potential role of diet and geographical distribution [15], this study was conducted to establish the same for the population that our lab has been catering to. Owing to its ease of use, transport, and comparative stability, dried blood spot has been used as the sample of choice for quantification of amino acids and for the screening and diagnosis of inborn errors of metabolism. The amino acid concentration between DBS and plasma among the neonates have already been compared in many studies and were found to be similar [16,17] . The reference intervals that were obtained from the DBS samples in this study also compared well with those obtained from plasma from other studies.

Even though diet of the mother has long been indicated as a potential factor which alters the amino acid concentration in the neonate [18], in our study, only methionine reflected a statistically significant difference between vegetarian and mixed diet. This may be attributed to the fact that most of the rich sources of methionine are animal based while most of the plant-based methionine (legumes, for example) are methionine-deficient in comparison [19]. That said, a full dietary history was not taken from the mothers and hence, a better picture would be obtained only after taking the diet history too into accordance.

The levels of tryptophan in the DBS of newborns who were born through normal vaginal delivery showed a statistically significant increase in comparison to those born through a caesarean section. We could not find any reason for this finding.

As far as we could confirm, a study for establishment of reference intervals for amino acids in the DBS of neonates have not been conducted in India. As such, this would be the first study to use HPLC to determine the concentration of amino acids in term neonates. Hence, we compared the values obtained in

**Table 4.** Biological reference interval for amino acids in DBS.

| Parameter | Mean (µmol/L) | Confidence interval for mean | | Standard deviation |
|---|---|---|---|---|
| | | Lower (µmol/L) | Upper (µmol/L) | |
| Aspartic acid | 61.5 | 58.2 | 64.8 | 21.9 |
| Glutamic acid | 77.4 | 71.8 | 83.1 | 37.4 |
| Asparagine | 69.8 | 65.8 | 73.8 | 21.9 |
| Serine | 135.6 | 127.7 | 143.5 | 51.9 |
| Glutamine | 604.1 | 568.4 | 639.8 | 235 |
| Glycine | 231.6 | 205.4 | 257.8 | 169.6 |
| Arginine | 85.1 | 75.61 | 94.5 | 62.1 |
| Threonine | 92.4 | 84.6 | 100.2 | 47.2 |
| Histidine | 96.6 | 90.1 | 103.4 | 43.7 |
| Tryptophan | 68.6 | 64.1 | 73.2 | 28.1 |
| Tyrosine | 65.9 | 62.4 | 69.5 | 23.5 |
| Alanine | 192.8 | 176.7 | 208.6 | 105.9 |
| Methionine | 70.6 | 65.1 | 76.1 | 36.2 |
| Valine | 112.1 | 104.3 | 119.9 | 50.1 |
| Phenylalanine | 60.3 | 56.9 | 63.8 | 21.1 |
| Isoleucine | 61 | 57.8 | 64.2 | 23.4 |
| Leucine | 82 | 70.9 | 93.1 | 73.7 |
| Lysine | 95.9 | 88.8 | 102.9 | 46.5 |

our study to two other studies done in Europe [20,21]. The first study had a sample size more than our study while the second study had a sample size much lesser than ours. Comparison of data between both the studies are shown in Table 5.

Two amino acids, namely proline and cysteine were not quantified in our study. This was because of difficulty in derivatization of the two, with proline peak not appearing in the chromatogram while cysteine peaks showed variable retention times. From the comparison, it is evident that the biological reference intervals for most amino acids are different in the population we are catering to when compared to the reference intervals established elsewhere in the world. This highlights the importance of establishment of a population specific reference interval to reduce the incidences of misdiagnosis.

Although every effort was taken to remove interfering factors from the study group, the authors identified a few limitations. They are listed below:

- ***Diet history of the mother***: A detailed diet history of the mother was not taken, which would have allowed to explain the variations based on the diet alone.
- ***Duration of storage of DBS cards***: Even though the samples were processed at the earliest, the storage duration might have been different for different samples. This may have caused variations in the measured parameters.
- ***Storage and transport of the DBS cards***: The DBS cards were stored under optimal conditions in the laboratory before processing of samples. But the storage conditions before the cards reaching the laboratory is unknown.
- ***Time of sample collection***: Most of the samples were collected from apparently normal neonates. Since there was no follow-up done on them, it is unknown whether they developed some diseases/metabolic disorders after the sample was taken.
- ***Difficulty in derivatizing proline and cysteine***: In the present study, we found it difficult to derivatize both proline and cysteine. No peak developed for proline while cysteine showed variable retention times. Hence, these two amino acids were not quantified in our study.

## Conclusion

Based on our study, the authors conclude that the reference intervals for amino acids in the DBS have a significant geographical variability, making it imperative that laboratories screening for aminoacidopathies either establish their own population specific reference intervals or adopt pre-established reference intervals. Our study, being the first of its kind in India, can serve as a reference for laboratories in our geographical area. Moreover, the study provides a template for other laboratories across the country to establish their own geography specific reference intervals for amino acids. Future studies can be conducted addressing the limitations of the patient studies and including sick babies to arrive at clinical decision cut offs and assessment of treatment response specific to our population.

**Table 5.** Comparison of amino acid reference intervals among similar studies.

| Amino acid | Current study (µM/L) | Nolasco D et al. [20] (µM/L) | Dietzun *et al.* [21] (µM/L) |
|---|---|---|---|
| Aspartate | 58.2 – 64.8 | – | 41.6 – 102.3 |
| Glutamate | 71.8 – 83.1 | 193 - 566 | 72.8 – 312.3 |
| Asparagine | 65.8 – 73.8 | – | – |
| Serine | 127.7 – 143.5 | 130 – 472 | 41 – 209.1 |
| Glutamine | 568.4 – 639.8 | 238 – 808 | – |
| Glycine | 205.4 – 257.8 | 182 – 637 | 47.2 – 247.3 |
| Arginine | 75.61 – 94.5 | <1 – 36 | 21.3 – 178.7 |
| Threonine | 84.6 – 100.2 | 66 – 415 | 15.1 – 138.5 |
| Histidine | 90.1 – 103.4 | 24 – 98 | 95 – 380 |
| Tryptophan | 64.1 – 73.2 | – | –– |
| Tyrosine | 62.4 – 69.5 | 34 – 541 | 18.3 – 126.2 |
| Alanine | 176.7 – 208.6 | 104 – 394 | 19.1 – 198.3 |
| Methionine | 65.1 – 76.1 | 10 – 39 | 20.5 – 142.3 |
| Valine | 104.3 – 119.9 | 46 – 224 | 30.0 – 126.2 |
| Phenylalanine | 56.9 – 63.8 | 30 – 97 | 7.2 – 82.2 |
| Isoleucine | 57.8 – 64.2 | 12 – 66 | 8.3 – 64.1 |
| Leucine | 70.9 – 93.1 | 31 – 130 | 22 – 105.8 |
| Lysine | 88.8 – 102.9 | 29 – 119 | 20.4 – 94.0 |
| Proline | – | – | 31.9 – 151.8 |

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## Declaration of Conflicting Interests

The authors do not report any conflict of interest.

## Authors' Contributions

AS concept design, designing the method, analysing the samples, and manuscript writing. VBS concept design, intellectual inputs, manuscript correction. LESL recruitment of patients, intellectual inputs. PCBW statistical analysis, intellectual inputs.

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