ORIGINAL ARTICLE

Reverse Phase-High Performance Liquid Chromatography: An Alternative to Expensive Tandem Mass Spectrometry Screening for Amino Acid Profiling in Dried Blood Spot in Resource Constrained Diagnostic Settings

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Abstract:

Background: Altered patterns of amino acid profiles are observed in various pathological conditions including nutrition related disorders, cancer, diabetes, urea cycle defects, mitochondrial respiratory chain disorders, and aminoacidopathies. *Aim and Objectives:* To develop a cost effective Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) method for the quantification of free amino acids in neonatal Dried Blood Spot (DBS) samples. *Material and Methods:* Free amino acids were extracted from neonatal DBS through elution with ice-cold methanol/2-mercaptoethanol. The extracted samples were then derivitized by using O-Pthalaldehyde (OPA)/2-mercaptoethanol, which resulted in the formation of OPA derivatives of aminoacids. These derivatives were quantified by RP-HPLC. The developed method was validated according to the International Conference on Harmonization ICH Q2 (R1) Guidelines and applied to clinically confirmed aminoacidopathies. *Results:* A good linearity was observed from 2.5 to 1000 μM. The between run imprecision (on two different days) though varied over a wide range for different amino-acids, the Coefficience of Variation (CV) was found to be less than 5% (range; 0.1 to 5% for different amino acids) and the recovery was found to be around 82-125% (for spiked amino acids). The quantified concentration of amino acids in plasma and DBS sample were found to be almost similar except for few amino acids like glycine, tyrosine, alanine, methionine and lysine which showed higher values in dried blood spot samples. DBS amino acid concentrations determined by RP-HPLC showed concordance to the concentrations obtained through Tandem Mass Spectrometry (TMS) of DBS/plasma. Reference intervals of amino acids were established in term neonates. The proposed method was applied to the analysis of samples obtained from confirmed aminoacidopathies. *Conclusion:* The present approach with DBS offers a valid alternative to the plasma/serum aminoacid profiling in HPLC or DBS in Tandem mass method, with advantages like lower sample volume, feasibility, affordability, improved specificity and can be attempted in a simple diagnostic setup with acceptable accuracy and precision.

Keywords: Aminoacid, Aminoacidopathy, Dried Blood Spot, Inborn Errors of Metabolism, Reverse Phase-High Performance Liquid Chromatography

Introduction:

Amino acid abnormalities are common in any pathological conditions (nutrition related disorders, cancer, diabetes, urea cycle defects, mitochondrial respiratory chain disorders, aminoacidopathies, organic acidemias, metabolic syndrome, cardiovascular disease) which affect metabolism [1-5]. Comprehensive analysis of

patterns of physiologic amino acids in blood, urine and cerebrospinal fluid helps in the early detection and prognostication of both Inborn Errors of Metabolism (IEM) and non-IEM disorders. Early detection of IEM especially helps in timely therapeutic intervention, which would prevent irreversible neurologic complications and halt or retard the progression of the disease [6]. The traditional sampling needs more volume of biological fluids which is undesirable in neonatal cohort. Present study is focused on Dried Blood Spot (DBS) sampling which is comparatively less invasive (compared to the insertion of cannula/ traditional blood sampling). Complete amino-acid profiling helps in both diagnosis and monitoring of various aminoacidopathies and respiratory chain disorders. However, amino acid profiling is usually done for highrisk cases for selected ten amino acids using mass spectrometry (gold standard) or complete profiling of amino acids by conventional ion exchange chromatography/aminoacid analyzer, however these techniques are expensive to employ in a resource limited scenario. The diagnostic importance of amino acid profile and the hassle free sampling of DBS necessitates the development of simple, precise, specific, accurate, cost-effective, and validated HPLC method which could serve as a basic modality to diagnose IEM at resource constraint settings, as many diagnostic settings are not equipped with sophisticated instrumentations. This study attempts to propose and validate a cost effective HPLC method for complete amino acid profiling (20 amino acids) in DBS samples which is comparatively less sensitive than mass spectra techniques but promises to provide a definite clue for the diagnosis of various IEM and non-IEM disorders.

Materials and Methods:

Reference standards of individual amino acids (99%), O-pthalaldehyde (⩾ 97%), β-mercapto-ethanol (99%), glacial acetic acid (99%), and triethanolamine (99%) of HPLC grade were obtained from Sigma Aldrich, Mumbai. Disodium tetraborate (99%), sodium hydroxide, sodium acetate trihydrate (98.5%), sample membrane filter-Durapore PVDF 45µM, solvent membrane filter-Nylon 0.45µm and Column, Purospher star RP-18E LP; 250 × 4.6mm, 5µm were obtained from Merck India Limited, Mumbai. HPLC grade methanol (99.8%) from Rankem was supplied by Aventor Performance Materials India Ltd, Thane, Maharashtra. DBS cards of Whatmann 903 were supplied by Perkin Elmer. HPLC grade water was obtained from Direct-Q Water Purification System (Merck Millipore India Pvt. Ltd).Working solution of OPA for derivatization was freshly prepared daily by dissolving 10 mg of OPA in 1ml of mixing stock. OPA reagent mixing stock (0.25M, pH-10.5 borate buffer: methanol: mercaptoethanol; 9:1:0.01) was found to be stable for 7 days, when stored at 8°C.

Instrumentation:

Chromatographic System:

RP-HPLC system, Schimadzu–UFLC Prominence (LC 20-AD) equipped with fluorescence detector (model-LC20AD) and data collection and processing in LC solutions (Lab Solutions) and PC was employed for the method development, validation and sample analysis for establishing reference range. The HPLC separation of the derivitized amino acids required two mobile phases. Mobile phase A was composed of 0.05M sodium acetate buffer with 0.1% triethanolamine adjusted to pH 6.8 using acetic acid, and mobile

Table 1: Particulars of the Concentration Gradient Flow

Timeline for Gradient Change (minutes)	Mobile Phase A (%)	Mobile Phase B (%)
0	90	10
0-5	80	20
5-10	74	26
10-25	64	36
25-35	50	50
35-40	25	75
40-50	10	90
50-60	10	90

phase B consisted of 100% HPLC grade methanol. All buffers were filtered through a 0.45-µm filter and degassed using sonicator and online degasser. All gradient changes were linear. The gradient conditions were tabulated in Table 1. The needle and loop of the manual injector was rinsed with 100% methanol in between injections.

Study Site:

The study was conducted at Centre of Excellence for Inborn Errors of Metabolism (CE-IEM), Kasturba Medical College Manipal, Karnataka, India after the approval from Institutional Ethics Committee (Ref: IEC 390/2017 dated 30th Aug 2017).

DBS Collection and Processing:

Residual samples (The leftover blood sample post its intended clinical analysis) of DBS specimens from newborn babies collected for a period of twelve months from 1st August 2017 (for the newborn screening for six metabolic disorders) were used for standardization and establishment of reference intervals in the term neonates. These DBS samples were collected on the third day of birth for the newborn screening test and dried at room temperature for three hours. Post screening, the residual samples of healthy neonates were de-identified and annotated with age, sex, and gestational age, date of birth and time of collection by laboratory in charge (who is not the part of the research team). The reversibly delinked samples were transferred to the envelopes with desiccants, transferred to zip lock plastic bags and stored at -80°C until further analysis (i.e. proposed study). The samples were reversibly delinked by the laboratory in charge (master data accessible only to the laboratory-in-charge). The reversible delinking of sample ensured tracing back the patient details in case any of the samples showed abnormal amino acid profiles which could ensure further diagnosis and management of the neonates. However, the individual informed consent was not taken as

newborn screening is a standard of care at the study site and the residual samples after NBS were utilized for the study.

To demonstrate the variability in the concentrations between plasma and DBS, residual blood samples sent for arterial blood gas analysis or ammonia levels analysis were used. Fifty µl of residual blood was spotted on Whatmann 903 filter paper and 250 µl of plasma was separated from the same sample for plasma amino acid profiling.

Dried Standard Spots Preparation:

Ten mmol/L amino acid standard solutions were prepared in 80% methanol and concentrated hydrochloric acid (20ml HCl/L of standard solution). Fifty µL of combined standard mixture, prepared by mixing equimolar individual standards is spotted on Whatmann 903 card and stored at -80°C until the day of analysis.

Storage Stability of Dried Blood and Standard Spots:

The twelve batches of aqueous aminoacid standards, DBS and dried standard spots of known concentration were stored at -80°C for 12 months and 30 days at -20°C. Also sample was tested for the stability with frequent (i.e. every 7^{th} day) freeze and thaw cycles. The stored samples were then run in triplicates according to the developed method to analyse the storage stability of the standards and dried blood spots.

Amino Acid Extraction Optimization from DBS:

Three mm punched disc of DBS /standard dried spot was mixed with 75µl of ice cold methanol for elution of free amino acid and centrifuged for 10 minutes at cold centrifuge with pre-temperature set at 4°C and 10000 rpm. The eluent were then mixed with 5 µl of β-mercaptoethanol and cold centrifuged for 5 minutes at 4°C and 10000rpm. This was followed by addition of 75µl of 80% ice cold methanol to the afore-obtained eluent for deproteinization and cold centrifuged for 10 minutes. β-mercaptoethanol reduces the disulphide bonds, denatures the proteins and also provides thiol group necessary for O-pthalaldehyde reaction with primary amines of amino acid.

Pre-column Sample Derivatization and RP-HPLC separation:

Hundred µl of supernatant was then subjected for OPA pre-column derivatization by addition of 25 µl of OPA reagent and made up to 1ml by adding 875 µl of start buffer (mixture of mobile phase A: mobile phase B; 5:1) followed by reversed phase HPLC separation as developed by Suresh Babu *et al*. [7]. Twenty µl of OPA derivatized DBS extract(OPA derivatives of amino acids) after sample filtration process were injected to the HPLC system equipped with C-18 column (250mm) and inbuilt incubator oven set at constant temperature, 40°C. Gradient (detailed in Table 1) of 0.05M sodium acetate buffer, pH 6.8 and methanol with flow rate of 1ml/minute was used. All gradient changes were linear. The needle and loop of the manual injector was rinsed with 100% methanol in between injections. Data on amino acid profile were determined fluorometrically with excitation and emission set at 352 nm and 435 nm respectively and at medium gain.

Complete separation was achieved within 45 minutes. Total analysis time, including derivatization (30 min), chromatography (45 min) and re-equilibration time with 90% methanol before the next sample injection (15 min) of the column, was 90 min.

Analytical Method Validation Parameters:

To validate the proposed method, analytical performance parameters such as linearity, accuracy, specificity, precision, and sensitivity (limit of detection and limit of quantitation) were determined according to the International Conference on Harmonization ICH Q2 (R1) and Food and Drug Administration (FDA) Guidelines. The proposed method was applied to clinically confirmed aminoacidopathies to validate the correlation between the calculated DBS amino acid concentrations from the standardized method to other published methods.

Specificity was determined by comparing the retention times obtained in the dried standard mixture spots with that of dried blood spots. Linearity was determined in the range from 2.5 to 1000 μM of dried standard mixture. Five concentration points of dried standard mixture (2.5, 5, 10, 50, 100 μM) were analysed. The trials were run in five replicates of individual concentration points to establish the linearity.

Accuracy was determined by spiking each amino acid standard individually and as a mixture of all standards with the three known concentrations separately to a known final concentration (baseline concentration of each aminoacid was quantified) in a dried standard spot/DBS. The average and individual recovery was analysed in three replicates for each spiked spots. Precision was determined as repeatability and intermediate precision. However, the other criteria of precision, establishing reproducibility data was found beyond the scope of the project. Five replicates of sample DBS with known concentration was processed for assessing repeatability and triplicates of the same were run on three consecutive days to assess the intermediate precision. Limit of detection and quantitation was determined based on signal to noise ratio and Relative Standard Deviation (RSD) of lowest limits of detection or quantification. Lowest limits of quantification and detection were determined by sequentially diluting the sample and standard of lowest concentration respectively and recording the RSD by running the method for six times (six replicates of the lowest concentration).

Reference Interval:

Residual reversibly delinked DBS of 250 healthy neonatal cohort (born at term gestation with normal APGAR score (>7) and birth weight >2500 g on exclusive breast-feeding) were recruited based on the Clinical & Laboratory Standards Institute guideline for establishing the reference interval. The power of the study was 80% with α-error of 0.05%. The recruitment of the samples was ensured by the geographic location (south west coastal Karnataka) and exclusion of low-birth weight, preterm babies, any sick neonate requiring medical intervention, underwent surgical procedure, congenital anomalies, chromosomal abnormalities, death or transfer to other hospital prior to discharge, family history of sibling death/IEM. Complete anonymization with appropriate annotation and delinking of samples were taken care by the lab in-charge. Results of our assessments with reference interval for healthy neonatal cohort were subjected to appropriate statistical analysis and reference range interval with 2.5^{th}–97.5^{th} percentile distributions was established.

Results and Discussion:

RP-HPLC method which was originally developed for quantitation of aminoacids in biological fluids like CSF, plasma and urine [7] was modified for its

extraction and quantification in DBS. The proposed method was standardized, optimized for the factors like the reduced reagent use for extraction of free aminoacids, concentration gradient of mobile phase and turnaround time, and validated for analytical performance parameters such as linearity, accuracy, specificity, precision, and sensitivity. The final optimized extraction and separation method is discussed under the methodology section (Amino acid extraction optimization from DBS; Pre-Column sample derivatization and RP-HPLC separation). The method could be used both for screening and follow-up confirmatory test. Free amino acids were extracted from DBS by eluting with ice-cold methanol/2 mercaptoethanol. The extracted samples were then derivitized by using O-Pthalaldehyde/2-mercaptoethanol, which resulted in the formation of OPA derivatives of aminoacids. These derivatives were quantified by RP-HPLC, and the developed method was validated for performance parameters according to the International Conference on Harmonization ICH Q2 (R1) Guidelines [8-10]. The proposed method was applied to screen and clinically confirm aminoacidopathies and also establish reference intervals healthy term neonates.

Storage stability of the samples; both standard and blood spots were attempted in present study along with the reference intervals for the individual aminoacids in the DBS healthy neonatal cohort. The attempt, though seems to be drifted from the focus of the study, was aimed to reproduce and sustain the proposed method in the resource constrained diagnostic settings. The aqueous aminoacid standards and DBS/dried standard spots were found to be stable at -80°C for 12 months. The stability of the same when stored for 30 days at -20°C was found to be lower. Also sample deterioration was observed with frequent freeze and thaw cycles while samples remained stable for 12 months at -80°C with minimal interference.

Retention time for individual amino acid was established following the optimized analytical condition for the peak identification. The area of the peak obtained for a given reference amino acid of known concentration has been used for final calculation of sample amino acid profile. Whenever there was ambiguity in deciding the given amino acid peak in the sample because of slight shift in retention time or tailing of the peak, the analysis was performed again after the sample was spiked with amino acid of interest [7].

The results of validation specifications of the optimized method are presented in Table 2, the results adhere to the specifications of fulfilling the acceptable criteria as per the guidelines of ICH and FDA [8-10]. The chromatographic separation of a dried amino acid standard spot and aqueous aminoacid standard (Aqueous Std. graph) are depicted in Fig. 1. The final concentration of each standard picked up in the aqueous amino acid standard graph was 200 Pico moles whereas for those of DBS i.e. the dried amino acid standard was 25 Pico moles (Reflected in Fig. 1, y axis- mV units in Std. graph and uV units in standard DBS graph). However, the difference in the peak areas of the equimolar aminoacids is explained by the difference in their relative fluorescence [7].

Table 2: HPLC Method Performance Characteristics for Amino Acid Profiling in DBS

Validation Parameters		Study findings	Acceptable criteria
Specificity		<±1	within ±3
Linearity (2.5 to 1000 µ M)		r^2= 0.999- 1	>±0.95
Accuracy	**Average recovery**	108%	90-110%
	Individual aminoacids	92-125 %	
Precision	**Repeatability and intermediate precision**	<5%	⩽5%
		Tau 5.7%	
		Orn- 7.1%	
LOD S/N ratio=3:1	**1-10 pmol/µL**	⩽9%	⩽10%
LOQ S/N ratio=10:1	**2.5-50 pmol/µL**	⩽ 8%	⩽10%

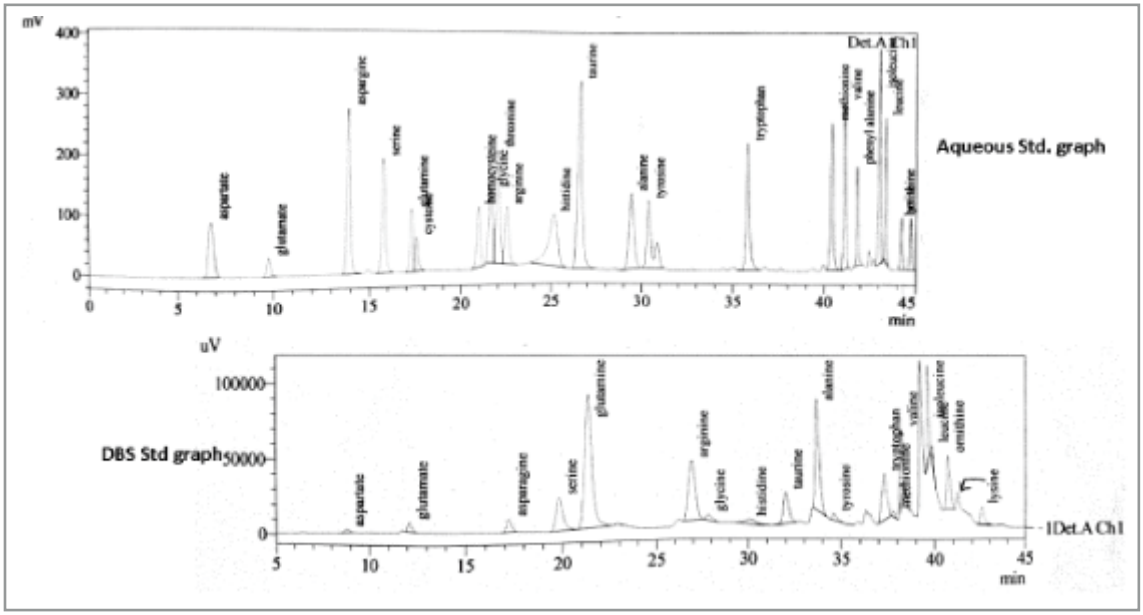

Fig. 1: Chromatogram of Aqueous Standard Mixture (Aqueous Std. graph) and Dried Standard Mixture (DBS Std. graph)

The method demonstrated good chromatographic separation of 20 amino acids. DBS amino acid concentrations determined by RP-HPLC showed concordance to the concentrations obtained through TMS of DBS. This established the acceptable corroboration between the calculated DBS amino acid concentrations from the standardized method to that of globally accepted method.

The stability of the individual aminoacid analytes in the polar mobile phase was decided based on previous studies and trial and error approach considering the factors like separation response due to their hydrophobic interaction and timely concentration gradient of mobile phase, thereby facilitating the elution. The stronger hydrophobic analytes with longer carbon chain, aromatic group like phenyl alanine, methionine, valine, isoleucine, ornithine and lysine are strongly bound to stationary phase and elute much later while the acidic aminoacids like aspartic acid, glutamic acid are less hydrophobic thereby eluting faster. The separation response of isoleucine, leucine, methionine, valine and phenylalanine were found to be deteriorating with time lapse. However, intermediate precision was <5% except in case of ornithine (7.1%), over a period of 10 months in stored unprocessed DBS sample and a week in DBS extract of aminoacids preserved at -80°C, provided the frequent freezing and thawing is evaded. Since, the samples were often stored to reach the adequate sample size for the amino acid profiling in conventional diagnostic laboratory, the separation response in an unprocessed and processed stored sample was studied to ensure the quality analysis. In addition, we observed that ornithine and lysine were significantly violating the acceptable intermediate- precision criteria. The between run imprecision (on three consecutive days) varied over a wide range for different aminoacids, however the CV for each recovered aminoacid was found to be less than or equal to 5% (range; 0.1 to 5% for different amino acids)except for Taurine (CV= 5.7%) and the accuracy for the recovery of individual aminoacids was found to be around 82-125% (for spiked amino acids). The average recovery of the aminoacid mixture was 108% which was well within the acceptable range [9]. These calculated validation specifications corroborated with those reported by others [6-8]. The concentration of amino acids in plasma and DBS sample were almost similar except for few amino acids like glycine, tyrosine, alanine, methionine and lysine which showed higher values in dried blood spot samples. The exceptional elevated concentrations in the DBS could be justified by the fact that the DBS is the dried whole blood cells which are involved in inter-organ transport of a few amino acids. Further their concentration in blood cells is obvious to be many-fold greater than the processed plasma [11].

The negligible difference (acceptable difference is ±3%) between the retention times of standards and samples as reported in Table 3 validates the claim of highly acceptable specificity of the peak identification [8]. Good linearity was observed between the concentrations of 2.5 to 1000 µM.

An important goal of both Millennium Development Goal-4 and Sustainable Developmental Goal-3 is improving the healthcare access to newborns and ensuring significant reduction in neonatal and under-five mortality [12]. The proposed method which was applied to the analysis of samples obtained from confirmed aminoacidopathies like phenylketonuria, tyrosinemia, and maple syrup

Table 3: Specificity Assessment

Amino Acids	Standard Solution (n=5)	Dried Blood Spot	Difference
	Retention Time (minutes)	Retention Time (minutes)	
Aspartate	10.45 ± 0.05	10.38 ± 0.01	-0.61
Glutamate	12.14 ± 0.23	12.20 ± 0.07	0.45
Asparagine	15.19 ± 0.31	15.23 ± 0.09	0.26
Serine	16.87 ± 0.30	16.98 ± 0.14	0.63
Glutamine	17.39 ± 0.30	17.51 ± 0.20	0.68
Arginine	19.74 ± 0.47	19.69 ± 0.36	-0.25
Glycine	20.92 ± 0.57	21.09 ± 0.51	0.81
Threonine	21.26 ± 0.65	21.40 ± 0.60	0.64
Histidine	22.67 ± 0.88	22.81 ± 0.76	0.62
Taurine	23.98 ± 1.11	24.11 ± 0.94	0.56
Tyrosine	24.93 ± 1.42	25.05 ± 1.21	0.46
Alanine	26.13 ± 1.37	26.25 ± 1.15	0.45
Tryptophan	34.61 ± 0.10	34.92 ± 1.09	0.90
Methionine	38.14 ± 0.04	38.07 ± 0.21	-0.20
Valine	40.22 ± 0.13	39.13 ± 0.62	-2.74
Phenylalanine	40.52 ± 0.03	40.34 ± 1.41	-0.46
Isoleucine	41.27 ± 0.06	40.81 ± 1.30	-1.12
Leucine	41.44 ± 0.01	41.24 ± 1.41	-0.46
Ornithine	41.92 ± 0.31	42.04 ± 1.08	0.29
Lysine	42.64 ± 0.51	42.87 ± 1.07	0.54

Values expressed in Mean ± Standard Deviation

urine disease guaranteed to achieve the developmental goals as it ensured early diagnosis and prognosis of treatable metabolic disorders. Result comparison with tandem mass spectrometry showed an appreciable concordance. This concordance emphasizes the potential efficacy and analytical utility of the proposed method in resource constrained diagnostics or the primary healthcare centers with makeshift laboratories to adapt the proposed method.

Table 2: RP-HPLC: Reference Intervals for Amino Acids in DBS

Aminoacids	Reference Interval in DBS (µM) (n=148)
Aspartate	3.956- 2367.88
Glutamate	48.68- 724.90
Asparagine	20.05-79.65
Serine	48.40-346.33
Glutamine	236.73-2884.90
Arginine	11.03-1051.95
Glycine	141.38-3210.33
Threonine	12.68-184.00
Histidine	22.35-186.00
Taurine	388.00-3701.65
Tyrosine	26.75-197.00
Alanine	64.40-1252.00
Tryptophan	46.70-4806.00
Methionine	7.35-1274.95
Valine	27.05-797.33
Phenylalanine	24.38-937.33
Isoleucine	20.00-465.00
Leucine	74.70-1442.33
Ornithine	9.00-538.00
Lysine	61.90-1087.00

Moreover, an additional outcome of this study was establishment of the reference intervals in the healthy neonatal cohort of native population (detailed in Table 4), so that, one does not have to derive a method specific reference range for interpretation of their results and can utilize the given reference ranges as a guide. However, the reference intervals established was that of the healthy neonatal cohort from the study location (South West Coastal Karnataka). Hence the study needs to be reaffirmed by subsequent studies as the sample size used in the study was inadequate considering the diverse geo-ethnic population. The geography specific interval though was not an objective of this study, the authors aimed to establish the one using the proposed method with the intention of repeatability and sustainability of the developed method.

However, the major limitation of our study was long turnaround time which could be resolved by employing the concept of quality by design like automation of aminoacid derivatization, standardization of time difference between the various steps, use of auto sampler. Automation would also resolve the differential degradation of OPA derivatives keeping the reaction time constant [8]. Further, increased automation would ensure the marked reduction of possible analytical errors [13]. The authors are working towards method optimization by these quality by design approaches. Finally, practical considerations of the proposed methodology when compared conventional methods have to be carefully gauged and assessed before engaging rapid transition.

Conclusion:

The present approach with DBS offers a valid alternative to the plasma/serum aminoacid profiling in HPLC or DBS in Tandem mass method, with advantages like lower sample volume, feasibility, affordability and improved specificity and can be attempted in a simple diagnostic setup with acceptable accuracy and precision.

References

1. Bi X, Henry CJ. Plasma-free amino acid profiles are predictors of cancer and diabetes development. *Nutr Diabetes* 2017; 7(3):e249.
2. Rodney S, Boneh A. Amino acid profiles in patients with urea cycle disorders at admission to hospital due to metabolic decompensation. *JIMD Rep* 2013;9:97-104.
3. Clarke C, Xiao R, Place E, Zhang Z, Sondheimer N, Bennett M, *et al*. Mitochondrial respiratory chain disease discrimination by retrospective cohort analysis of blood metabolites. *Mol Genet Metab* 2013;110(1-2): 145-52.
4. Wasim M, Awan FR, Khan HN, Tawab A, Iqbal M, Ayesha H. Aminoacidopathies: prevalence, etiology, screening, and treatment options. *Biochem Genet*. 2018;56(1-2):7-21.
5. Hu W, Sun L, Gong Y, Zhou Y, Yang P, Ye Z. Relationship between branched-chain amino acids, metabolic syndrome, and cardiovascular risk profile in a Chinese population: a cross-sectional study. *Int J Endocrinol* 2016; 2016: 8173905.
6. Shetty PP, Nagendra K, Nalini K, Rao P, Bhaskaranand N, Belle VS. Amino acid profile and lactate pyruvate ratio – potential adjunct markers for differentiating inborn errors of metabolism. *Indian J Clin Biochem* 2019; 35(4):430-435.
7. Babu SV, Shareef MM, Shetty AP, Shetty KT. HPLC method for amino acids profile in biological fluids and inborn metabolic disorders of aminoacidopathies. *Indian J Clin Biochem* 2002; 17(2):7-26.
8. Bartolomeo MP, Maisano F. Validation of a reversed-phase HPLC method for quantitative amino acid analysis. *J Biomol Tech* 2006; 17 (2):131-137.
9. Shabir GA. Step-by-step analytical methods validation and protocol in the quality system compliance industry. *J Validation Technol* 2004; 10(4): 314-324.
10. Reason AJ. Validation of amino acid analysis methods. In: B.J Smith, editors. Methods in Molecular Biology. Protein Sequencing Protocols, 2nd ed. Humana Press Inc., Totowa, NJ, 2003; 211: 181-194.
11. Lewis MA, Waterhouse C, Jacobs SL. Whole-blood and plasma amino acid analysis: gas-liquid and cation-exchange chromatography compared. *Clin Chem* 1980; 26(2): 271-276.
12. Neogi SB, Zodpey S. Newborn Care in India: Where do we stand in 2015? *J Krishna Inst Med Sci Univ* 2015; 4(2): 1-9.
13. Dhotre PS, Dhotre SV, Shaikh AKAR. A Comparative Study of Pre-analytical Errors in Central Clinical Laboratory in a Tertiary Care Hospital in Maharashtra. *J Krishna Inst Med Sci Univ* 2020; 9(2): 67-72.

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