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Research Article

Reference Intervals of Amino Acids and Acylcarnitines in the Blood Spot by Tandem Mass Spectrometry for Use in a Newly Established Extended Newborn Screening Program in the Fars Province South West of Iran

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Abstract

Objective: Determine reference intervals for amino acids and acylcarnitines in blood spot of newborns from Fars province south west of Iran for further use in diagnosis of inborn errors of metabolism.

Methods: One thousand Healthy neonates 1-5 days of age were included in this study. The reference intervals for amino acids, acylcarnitines in newborn dried blood spots using MS/MS conducted in 1000 healthy neonates from Fars Province, cities and villages from South West of Iran were determined based on the 1 and 99 percentiles of 1000 samples.

Results: Thirty-eight analytes that allow the diagnosis of more than 40 inherited metabolic disorders were tested. The LC-MS/MS method for analysis of amino acids and acylcarnitines was validated that to be linear and precise. The reference intervals were determined for amino acids and acylcarnitines that are used in the further newborn screening tests in the Fars region.

Conclusion: The study has contributed to present the usual concentration levels of amino acids, acylcarnitines that could be used as reference for newborn metabolic screening program in south west of Iran for the first time in the region.

Keywords: Neonatal Screenings; Inborn Errors; Amino Acids; Acylcarnitines; Reference Intervals

Introduction

Inborn errors of metabolism (IEM) are heterogeneous group of genetic disorders caused by a defect in a metabolic pathway, leading to the accumulation of toxic intermediate metabolites can result in death at an early age, creating a considerable family and social burden. Inborn errors of metabolism with nonspecific clinical manifestations and can be presented at any age, complicating diagnostic evaluations. The consequences are severe, causing morbidity and mortality in pediatrics clinical practice [1]. Inborn errors of metabolism are present in all ethnic groups and across every age but the incidence of these disorders is higher in the Middle East due to consanguinity. In the Fars region there has been a delay in diagnosis or misdiagnosis of newborn with inherited metabolic disorders and lack of treatment for newborns due to the lack of specialized laboratories that perform accurate tests. Recently the extended Newborn Screening Program for inherited metabolic disorders has been established in the Fars Province south west of Iran as a part of mandatory program and MS/MS is routinely used for newborn screening in this region and we have established a

Newborn screening laboratory in the region using Tandem mass spectrometry (MS/MS) as a diagnosis tool for inborn errors of metabolism capable of detection and quantitation of amino acids and acylcarnities with high sensitivity, specificity, accuracy and precision [4] in detecting over 50 different inherited metabolic conditions [5]. Because of the urgent need for a highly sensitive method for diagnostic and efficient inborn errors of metabolism screening in south west of Iran this study is focused on the establishment of reference intervals for the first time for amino acids and acylcarnitines in the samples of Iranians newborns in the Fars province in the south west of Iran using MS/MS technology.

Materials and Methods

Subjects

Blood spot samples of 1000 healthy newborns from cities and villages in the Fras Province South west of Iran were collected between April to May 2019 used in the study. According to the standards from Clinical and Laboratory Standard Institute [20] blood specimens were collected by heel-stick and spotted on filter paper which were dried for 24 hours at room temperature and then stored at 4° C until use. As a part of the Newborn Screening program policy in the region written informed consent was obtained from the mother or father of each newborn participated in the newborn screening program in the Fars Province.

Inclusion and exclusion criteria

The newborns included in this study were not diagnosed and suffering from any disorder or disease and have weights in the range of 2.5kg -4kg, gestational ages of 37-42 weeks and positive newborns diagnosed with inherited metabolic disorders were excluded from this study.

Materials

Isotopically labeled internal standards amino acids and acylcarnitines were used from Cambridge isotopes laboratory. Dried blood samples were analyzed using Waters Alliance (2795 separation module) coupled with Waters Quattro micromass Tandem mass spectrometer. Acetonitile, methanol and water LC/MS/MS grade were purchased from Merck company.

Sample preparation

Dried blood spots (DBS) were punched at 3.2 mm diameter and placed into a single well of a polystyrene 96-well plate with the addition of 100 ul internal standards of amino acids and acylcarnitines. The plate was sealed and shaken at 750 rpm for 20 min at 25º C. The extracts were transferred to a new polystyrene 96-microwell and placed into an autosampler tray for MS/MS analysis.

MS/MS analysis

A triple quadrupole tandem mass spectrometer, operated in positive-ion mode was used for analysis of amino acids and acylcarnitines. The samples were run using gradient method with mobile phase (acetonitrile: water - 50:50 containing 0.05% formic acid).

Linearity

Linearity of the method for amino acids and acylcarnitines was estimated in duplicated by inter-assay analysis of the dried blood spots calibrators enriched at four different concentrations very low, low, intermediate and high. Coefficients of linear regressions (R values) were determined.

Precision and accuracy

Intra-assay and inter-assay precision amino acids and acylcarnitines were determined at two medium concentration levels of the dried blood spots calibrators. Intra-assay precision was determined by duplicates in five injections, whereas the inter-assay precision was determined in duplicates for 5 days and two different analysts.

Results

Validation of the method

Coefficients of linear regressions of amino acids and acylcarnitines were >0.96 for all analytes (Table 1). The mean intra-assay precision (Table 2) and inter-assay precision (Table 3) over the entire concentration range was less than 20%.

Concentrations of AAs, ACs in the studied population

In 100 healthy neonates a total of 37 analytes were used to determine the reference intervals. Specific markers were selected according to information obtained from Regions 4 Genetic New Born Screening [22].

Glutamic acid (288.8 umol/l), Alanine (268.2 umol/l), glycine (204.1 umol/l) and Leucine (101.1 umol/l), and Arginine (15.6 umol/l) citrulline (10.2 umol/l) and for acylcarnitine.

The reference intervals of 37 amino acids and acylcarnitines presented in table 4. In general, the mean amino acids concentrations ranged between10.2 and 288.8 µmol/L. The most abundant amino acids were Glutamic acid (288.8 µmol/L), Alanine (268.2 µmol/L), glycine (204.1 µmol/L) and Leucine (µmol/L) whereas Arginine (15.6 µmol/L) and citrulline (10.2 µmol/L) were the less abundant.

In general, acylcarntines with shorter chains were the most concentrated whereas those of longer chain were the less abundant. Higher concentrations were observed for free C0 (16.1 µmol/L), C2 (14.4 µmol/L) whereas C12:1 (0.01 µmol/L) C18:2 (0.01 µmol/L) C18OH, C18:1OH (0.02 µmol/L) and C16OH, C16:1OH (0.03 µmol/L) were the less abundant (Table 4).

Discussion

In this study the reference intervals of amino acids and acylcarntines in the blood spots are reported for the first time in the Fars province, South West of Iran associated with more than 40 inherited metabolic disorders using automated tandem mass spectrometry (MS/MS) on dried blood spots collected from newborns in the Fars province for the establishment of normal values for the analyzed metabolites that meets the requirements of the Clinical and Laboratory Standards Institute (CLSI) guidelines [24]. Results indicated that blood samples enriched with each analyte in filter paper spots subsequently mixed with labelled internal standards led to data linearity over a wide range of concentrations.

Validation parameters established for this method displayed great precision and accuracy leading to CVs according to internationally accepted values for bio analytical methods to be considered reliable and reproducible [21]. In comparison with 1st and 99th percentile reported in Region 4 genetics for these metabolites, we found values in similar ranges for most of them using a much smaller population. Use of MS/MS for Newborn screening of inherited metabolic disorders offer some advantages such as analytical sensitivity, selectivity and accuracy, with the possibility to measure several analytes in a single analysis and relatively low rate of interferences [29]. Most of the limitations of the method presented here are related to the dried blood spot, blood sampling the volume of blood contained in the blood spot punch, can be solve by strict adherence to the general standard guidelines [20]. In this study the transportation and storage conditions for 1000 dried

Analyte	Concentrations in µmol/L				
	Very Low	Low	Intermediate	High	Linearity R2
Alanine	146.5	205.0	215.5	271.4	0.9834
Arginine	9.52	112.4	228.4	285.6	0.9828
Aspartic acid	2.2	4.5	6.61	10.71	0.9731
Citrulline	14.6	33.4	61.7	118.6	0.9734
Glutamic acid	73.9	98.1	117.2	176.8	0.9779
Glycine	149.5	123.7	339.3	507.3	0.9881
Leucine	81.9	156.6	253.3	371.7	0.9898
Methionine	39.0	53.2	82.6	113.7	0.9718
Ornithine	65.0	168.8	204.2	223.6	0.9721
Phenylalanine	33.6	115.3	199.1	251.9	0.9904
Tyrosine	31.6	161.3	319.8	419.2	0.993
Valine	75.1	205.9	297.7	351.9	0.9666
C0-Carnitine	13.9	26.0	37.4	46.2	0.9950
C2-Carnitine	9.0	19.0	26.3	32.3	0.9865
C3-Carnitine	0.5	3.1	6.5	10.4	0.9922
C4-Carnitine	0.1	0.9	2.1	3.5	0.9876
C5-Carnitine	0.14	0.6	1.6	2.9	0.9600
C6-Carnitine	0.1	0.5	1.0	2.2	0.9540
C5DC -Carnitine	0.2	0.3	0.61	1.73	0.9619
C5OH -Carnitine	1.20	2.9	1.0	4.88	0.9716
C8-Carnitine	0.03	0.6	1.1	2.50	0.9645
C10-Carnitine	0.07	0.8	1.3	3.4	0.9962
C12-Carnitine	0.02	1.1	2.1	3.3	0.9996
C14-Carnitine	0.03	0.7	1.7	3.3	0.9637
C16-Carnitine	0.66	4.0	7.5	9.7	0.9908
C16OH-Carnitine	0.05	0.3	1.3	1.5	0.9621
C18-Carnitine	0.56	1.6	3.3	4.9	0.9901
C18OH -Carnitine	0.03	0.2	0.84	1.15	0.9659

Table 1: Linearity.

Analyte	Coefficient of Variation (%)			Concentrations in µmol/L		
	Low	Intermediate	High	Low	Intermediate	High
Alanine	19.6	9.8	14.5	205.0	215.5	271.4
Arginine	13.5	7.9	9.19	112.4	228.4	285.6
Aspartic acid	13.9	12.5	9.20	4.5	6.61	10.71
Citrulline	15.8	9.3	10.9	33.4	61.7	118.6
Glutamic acid	19.3	4.7	2.9	98.1	117.2	176.8
Glycine	18.7	15.9	12.9	123.7	339.3	507.3
Leucine	4.5	12.9	9.1	156.6	253.3	371.7
Methionine	10.2	13.5	13.2	53.2	82.6	113.7
Ornithine	6.9	9.7	15.9	168.8	204.2	223.6
Phenylalanine	8.6	10.4	10.3	115.3	199.1	251.9
Tyrosine	15.6	8.9	12.1	161.3	319.8	419.2
Valine	6.9	12.8	8.10	205.9	297.7	351.9
SUAC	14.77	7.9	5.6	2.5	3.7	4.3
C0-Carnitine	11.9	13.1	10.7	26.0	37.4	46.2
C2-Carnitine	15.0	10.5	6.8	19.0	26.3	32.3
C3-Carnitine	17.3	12.2	9.4	3.1	6.5	10.4
C3DC-Carnitine	13.3	28.8	11.4	0.8	1.4	3.2
C4-Carnitine	12.0	8.3	8.4	0.9	2.1	3.5
C5-Carnitine	15.8	12.8	10.9	0.6	1.6	2.9
C6-Carnitine	16.6	14.0	13.8	0.5	1.0	2.2
C5DC-Carnitine	18.7	20.3	8.89	0.3	0.61	1.73
C5OH-Carnitine	18.2	16.0	13.16	2.9	1.0	4.88
C8-Carnitine	14.0	19.6	4.22	0.6	1.1	2.50
C10-Carnitine	14.2	14.5	8.8	0.8	1.3	3.4
C12-Carnitine	19.2	12.3	8.2	1.1	2.1	3.3
C14-Carnitine	19.2	18.0	6.6	0.7	1.7	3.3
C16-Carnitine	11.3	12.5	6.4	4.0	7.5	9.7
C16OH-Carnitine	16.8	15.4	12.2	0.3	1.3	1.5
C18-Carnitine	11.1	4.1	11.9	0.56	1.6	3.3
C18OH-Carnitine	14.0	12.4	12.7	0.2	0.84	1.15

Table 2: Intra-assay precision at three concentrations (low, intermediate, and high). N = 8.

Analyte	Coefficient of Variation (%)		Concentrations in µmol/L	
	Low	High	Low	High
Alanine	10.6	20.7	482.1	777.9
Arginine	14.1	15.6	89.1	287.9
Citrulline	13.2	11.5	27.2	146.5
Glycine	12.1	9.6	452.6	1224.6
Leucine	7.8	14.6	186.7	555.3
Methionine	8.5	6.1	53.4	112.6
Ornithine	6.8	12.5	159.4	239.0
Phenylalanine	6.8	6.7	128.1	319.5
Tyrosine	10.5	7.7	187.1	508.3
Valine	6.9	8.6	230.7	457.4
C0-Carnitine	13.6	13.4	25.9	52.3
C2-Carnitine	10.4	11.4	20.3	40.5
C3-Carnitine	10.0	10.8	4.8	12.5
C4-Carnitine	10.6	10.2	0.93	4.9
C5-Carnitine	10.6	9.9	0.58	3.3
C6-Carnitine	9.3	10.1	0.46	2.5
C5DC-Carnitine	9.7	9.5	0.57	1.8
C5OH-Carnitine	9.3	8.9	1.3	4.1
C8-Carnitine	9.8	4.1	0.46	2.4
C10-Carnitine	9.1	3.5	0.64	3.3
C12-Carnitine	9.8	6.8	1.1	3.5
C14-Carnitine	9.1	7.2	0.60	3.6
C16-Carnitine	9.6	8.2	3.9	10.9
C16OH-Carnitine	10.2	7.9	0.25	1.6
C18-Carnitine	9.6	5.2	1.5	4.8
C18OH-Carnitine	9.3	3.8	0.17	1.2

Table 3: Inter-assay precision at two concentrations (low and high). N = 8.

Metabolite	Mean	SD	PERC 1	PERC 99
Alanine	268.2	89.1	100.0	458.9
Arginine	15.6	5.1	3.9	31.3
Citrulline	10.2	4.7	1.6	25.8
Glutamic Acid	288.8	95.6	72.5	472.3
Glycine	204.1	108.7	23.4	536.0
Methionine	23.8	8.6	5.7	41.8
Phenylalanine	36.8	12.3	19.6	74.1
Tyrosine	84.9	36.8	32.3	214.7
Valine	73.9	29.5	77.9	187.9
Leucine	101.1	28.4	39.9	176.7
Ornithine	55.5	24.1	10.4	133.3
C0	16.1	7.1	6.0	39.4
C2	14.4	7.3	5.1	37.6
C3	2.1	1.0	0.4	5.4
C4	0.4	0.2	0.01	0.86
C5:1	0.11	0.07	0.01	0.34
C5	0.19	0.12	0.01	0.70
C6	0.11	0.07	0.01	0.34
C8	0.06	0.03	0.01	0.140
C10	0.24	0.12	0.01	0.47
C12	0.18	0.13	0.01	0.64
C14	0.18	0.08	0.04	0.40
C16	4.7	1.53	0.54	7.66
C18	0.58	0.30	0.21	1.58
C5DC	0.28	0.12	0.01	0.53
C5OH	0.17	0.08	0.04	0.40
C16OH	0.03	0.01	0.01	0.06
C16:1OH	0.03	0.03	0.01	0.170
C18OH	0.02	0.01	0.01	0.06
C18:1OH	0.02	0.02	0.01	0.09
C10:1	0.156	0.08	0.01	0.350
C10:2	0.08	0.05	0.01	0.24
C14:2	0.07	0.03	0.01	0.12
C14:1	0.04	0.02	0.01	0.10
C18:2	0.01	0.03	0.01	0.14
C18:1	1.56	0.88	0.11	3.86
C12:1	0.06	0.04	0.01	0.16

Table 4: Reference intervals of amino acids and acylcarntines.

SD: standard deviation

PERC 1: Percentile 1 lower limit

PERC 2: Percentile 99 upper limit

blood spot samples were fully controlled as we already reported that the integrity of dried blood spots can be compromised within a short time frame by humidity and temperature during transpor-

tation of the samples, a significant degradation of AAs and ACs in the blood spot at high temperature (45° C) and humidity (>70%), particularly in the first day of storage [31].

Conclusion

In conclusion, this is the first report of analyses of amino acids and acylcarntines for inborn errors of metabolism diagnosis by MS/MS in south west of Iran despite all the difficulties and challenges. Data reported here represents a significant contribution to establish normal concentration levels of amino acids and acylcarnitines be used as reference for implementation of a new newborn metabolic screening program in Iran based on MS/MS technology, allowing to define the national prevalence of inherited metabolic disorders.

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