Establishment and Validation of Reference Values for Amino Acids and Acylcarnitines in Dried Blood Spots for Omani Newborns Using Tandem Mass Spectrometry

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Received: 12 November 2021

Accepted: 11 April 2022

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DOI 10.5001/omj.2022.88

Abstract

Objectives: The objectives of this study were to establish a reference range for acylcarnitines (ACs) and amino acids (AAs) concentrations in dried blood spot (DBS) samples of Omani neonates and to evaluate the effect of age and gender on ACs and AAs.

Methods: An electrospray-ionization tandem mass spectrometry (+ESI-MS/MS) was used to determine ACs and AAs concentrations in DBS samples collected from 1302 healthy newborns (0-7 days) delivered at Sultan Qaboos University Hospital (SQUH) between August 2008 and May 2009.

Results: More than fifty biomarkers that allow diagnosis of various inborn errors of metabolism (IEMs) were measured and the 1st and 99th percentile values were determined and compared with published international data. In comparison with the 1st and 99th percentile reported by Collaborative Laboratory Integrated Report (CLIR), our results were comparable despite a much smaller sample size. We found that age had a significant effect on most ACs and AAs except decadienoylcarnitine (C10:2), decenoylcarnitine (C10:1), adipylcarnitine (C6-DC), palmitoylcarnitine (C16:1), steatoylcarnitine (C18), tyrosine (Tyr), phenylalanine (Phe) and valine (Val). Whereas, gender of the neonate had insignificant effect on most of ACs and AAs except C0, acetylcarnitine (C2), hexanoylcarnitine (C6), octanoylcarnitine (C8), malonylcarnitine (C3-DC), C10, dodecenoylcarnitine (C12:1), dodecanoylcarnitine (C12) and tetradecanoylcarnitine (C14:1).

Conclusions: MS/MS is a highly effective tool for high throughput screening of IEMs. This study is the first to publish reference intervals for ACs and AAs from DBS samples of Omani newborns. The results may prove to be of significance when determining cut off values that maybe considered for newborn screening in the near future.

Keywords: Oman; Tandem Mass Spectrometry; MS/MS; Inborn Errors of Metabolism; Reference Range.

Introduction

IEMs are a rare group of genetic disorders that can produce serious clinical consequences for an affected neonate or young infant.[1] Basically, IEMs are permanent biochemical disorders that are caused by deficiency of a functional enzyme, transmembrane transporters or similar proteins, which then results in blockage of the corresponding metabolic pathway. This consequently leads to accumulation of metabolites prior to the metabolic block, and/or deficiency in the ultimate products of the pathway.[2] Late diagnosis may result in severe, though variable, consequences that may include mental retardation, physical disability, neurological damage or even death.[1] A significant number of treatable IEMs are readily diagnosed through basic metabolic investigations, which include the following: lactate, ammonia, amino acids in plasma, urinary organic acids, and acylcarnitine profiles.[3]

Tandem mass spectrometer (MS/MS) is a powerful technique for neonatal screening for IEMs. It allows simultaneous detection and identification of multiple analytes with high sensitivity and specificity in a single analytical run from one disk of dried blood. MS/MS is capable of detecting over 50 different conditions.[4] This capability makes MS/MS comprehensive, versatile and effective when used for massive screening.[5] MS/MS

currently replaces traditional single-analyte screening techniques that usually measure an individual biomarker for each disease. At present, MS/MS is routinely used in many clinical biochemical laboratories across the globe.[6-10]

Because of the importance of the presence of rigorous reference range for AAs and ACs in MS/MS, this study is focused on the establishment of concentration values of AAs and ACs in Omani newborns population using derivatized non-kit ESI-MS/MS method. The influence of age and gender on ACs and AAs has been documented.[11-14] Therefore, the effect of these two factors on DBS ACs and AAs concentrations was evaluated in this study.

Methods

The newborns included in this study were Omani healthy males and females delivered at SQUH between August 2008 and May 2009. They were all delivered at term (gestational age 37 – 42 weeks), appropriate for gestational age (weight of 2500 – 4000 g), and required no active resuscitation at birth (APGAR score was more than 7 at 1 minute & 5 minutes). There was no clinical evidence of congenital anomalies on routine newborn examination. Blood specimens were collected by heel prick 24 hours to 7 days after birth. The blood was spotted on Scheicher and Schuell (S & S 903™, Dassel, Germany) filter paper card and allowed to dry at room temperature. The dry card was then sent to the laboratory and stored at 4 ºC until analysis. The healthy newborns were divided into 3 groups according to age, namely, group1: <48 hours (N=1026, 78.8%), group 2: 48-72 hours (N=171, 13.1%) and group3: >72 hours (N=105, 8.1%). This study is only a retrospective review of previously collected electronic records pertaining to the analytes of interest. The consent of subjects/care givers involved in the original pilot study were obtained then. This study retrospectively reviews the analytes saved in the laboratory database without any patient contact or involvement. This research project was approved by the Medical Research Ethics Committee (MREC), College of Medicine and Health Sciences, Sultan Qaboos University.

Isotopically- labelled internal standard of amino acids and acylcarnitines were purchased from Cambridge Isotope Laboratories (Andover, USA), 3 M butanolic HCl was purchased from Regis Technologies (Morton Grove, USA), HPLC-grade acetonitrile and methanol were purchased from Sigma-Aldrich (Steinheim, Germany) and VWR International (Geldenaaksenban, Belgium) respectively. Polypropylene 96-well microtiter plates were purchased from Corning Inc. (Corning, USA). Base, low, medium and high level blood spot controls were supplied by the Newborn Screening Quality Assurance Program of Center of Disease Control and Prevention (CDC). Dry blood samples (DBS) were analyzed using Waters ACQUITY UPLC™ system coupled to tandem mass spectrometer TQD (Micromass UK limited, Manchester, UK).

A 3.2 mm punch of the dried blood spots (DBS) were placed into a single well of a 96-well polystyrene plate. Then, 100 µL of a working solution containing internal standards of AAs and ACs in methanol was added. The plate was sealed and shaken for 30 min at 300 rpm. The extract was transferred to a new polystyrene 96-well plate and dried on heating block at 45 ºC under air flow for approximately 30 min. Then, 60 µL of butanolic HCl was added, the plate was sealed with adhesive film and incubated at 65 ºC for 15 min. The mixture was again dried for about 45 min. Finally, the residue was reconstituted with 100 µL of mobile phase [80: 20 (v:v) (acetonitrile: water)], covered with aluminum foil, shaken for 5-10 min at 300 rmp and loaded into an autosampler for MS/MS analysis.

The Waters ACQUITY® TQD was operated in positive ionization mode. The capillary voltage, source temperature, desolvation temperature, nebulizer nitrogen flow rate and desolvation nitrogen flow rate were set at 3.20 kV, 125 ºC, 250 ºC, 65 L/h and 550 l/h respectively. The samples were run using isocratic mobile phase consists of 80: 20 (v:v) (acetonitrile: water). The samples were injected directly into tandem mass spectrometer via a pipeline connected to the LC without chromatographic separation. The Flow rate was 300 µL/min and 20 µL of sample was injected. The total run time was 1.7 min per sample. The analysis was done using sex different scanning functions per run: 1. Precursor ion of m/z 85 (scan range m/z 210 – 505) for ACs, 2. Product ion of m/z 459 (scan range m/z 65 – 150), 3. Neutral loss of m/z 102 (scan range m/z 125 – 270), 4. Neutral loss of m/z 102 (scan range m/z 170 - 270), 5. Neutral loss of m/z 102 (scan range m/z 230 - 250), Neutral loss of m/z 102 (scan range m/z 130 - 150). The Masslynx™ version 4.1 and Neolynx softwares (Waters Corp., MA, USA) were used for data acquisition, analysis and interpretation. The quantification of target analytes was achieved by calculating the ion abundance ratios of each pure compound relative to isotopically labeled internal standards (IS).

Data were statistically analyzed using Statistical Package for the Social Science (SPSS) version 23 (IBM, NY, USA). SPSS version 23 was used to calculate 1st, 5th, 10th, 90th, 95th and 99th percentile of each ACs and AAs. A Shapiro-Wilk Test was used to check normal distribution of data. Correlations between age, gender and each analyte concentration were tested with a Spearman test. A p value ≤ 0.05 was considered significant.

Results

A total of 1302 healthy Omani neonates between 1 and 7 days of age, 674 males (51.8%) and 628 females (48.2%) were screened in this study. The newborns in this study were assigned to three age groups as described earlier. Early hospital discharge of newborns was a concern as healthy newborns delivered at SQUH generally get discharged after 24 hours of normal delivery. DBS samples were collected as late as possible prior to discharge. The (average ±SD) age at collection was 40 ± 29 hours. Out of the 1302 newborns studied, only six were tested at age younger than 24 hours of life, 18 hours (n=1), 20 hours (n=1), 21 hours (n=3) and 22 hours (n=1) provided that the newborn has had breast or normal infant formula feeding prior to the sample collection. Since none of the variables appeared visually as a notable outlier on the scatterplot, data from these six newborns were included in the determination of the percentiles.

Concentrations of 28 ACs and 11 AAs were measured using MS/MS. In general, short-chain ACs were the most abundant. Higher concentrations were observed for free-carnitine (C0) (mean= 24.04 µM) and acetylcarnitine (C2) (mean= 16.14 µM), whereas hydroxyoctadecanoylcarnitine (C18-OH) was the least abundant acylcarnitine with mean concentration of (0.02 µM). Hexadecanoylcarnitine (C16) was the most abundant among all long-chain acylcarnitines with mean concentration of (3.23 µM). With regards to AAs, glycine (Gly) was found to be the most abundant AA (mean = 244.76 µM), while arginine (Arg) was the least abundant with mean concentration of 9.38 µM.

Normal Concentrations of ACs and AAs were evaluated for correlation with age and gender. All data sets showed a normal distribution when they were evaluated by Shapiro-Wilk test for normality. Therefore, Pearson correlation test was used to check for the behavior of various concentrations of ACs and AAs with respect to these two factors. All analytes were significantly affected by age except for C10:1, C10:2, C6-DC, C16:1, C18, Tyr, Phe and Val. The effect of age was not consistent with all analytes. The age of newborns was positively correlated with C0, C2, isovalerylcarnitine (C5), hydroxyisovalerylcarnitine (C5-OH), methylmalonylcarnitine (C4-DC), hydroxyhexadecanoylcarnitine (C16-OH), octadecenoylcarnitine (C18:1), C18-OH, Alanine (Ala), Proline (Prol), Leucine + isoleucine (xLeuc), Methionine (Met), Ornithine (Orn), Citrulline (Cit), Arg and Gly, whereas it was negatively correlated with propionylcarnitine (C3), butyrylcarnitine (C4), tiglylcarnitine (C5:1), C6, C8, C10, C12:1, C12, C14:1, C16, C18 and C18:1-OH. Gender, however, had significant effect on C0, C2, C6, C8, C3DC, C10, C12:1, C12 and C14:1. In all analytes that were significantly correlated with newborn's gender, concentrations were higher in males than females.

Our ACs and AAs' 1st, 10th, 50th, 90th, and 99th percentile values were compared with Collaborative Laboratory Integrated Reports (CLIR) reference ranges,[15] which are derived by retrospective analysis of large data points from a growing worldwide community of collaborators. Although the sample size used in our study is small compared to CLIR, the 99th percentile values for ACs and AAs are comparable and are substantially different from the currently adopted reference ranges in our laboratory, which were based on newborn screening study carried out in Saudi Arabia in which 5000 healthy neonates were screened and the reference ranges assigned as 0.5% (bottom) and 99.5% (top) (Table 1).

Table 1: Reference concentration values for individual acylcarnitines and amino acids with selected commonly used ratios in dried blood spots of healthy neonates using MS/MS

Marker or	**New SQUH RR**			**Old SQUH RR**		**CLIR RR**		
Ratio	**N**	**P_1**	**P_{99}**	**Bot**	**Top**	**N**	**P_1**	**P_{99}**
C0	1302	10.34	56.9	**6.0**	**72.0**	2924K	9.73	49.0
C2	1302	1.97	52.10	**0.0**	**74.0**	2798K	8.42	47.6
C3	1302	0.58	6.09	**0.0**	**10.0**	5028K	0.60	4.01
C4	1302	0.10	0.92	**0.0**	**1.80**	2882K	0.10	0.57
C5:1	1302	0.01	0.09	**0.00**	**0.50**	1726K	0.01	0.06
C5	1302	0.05	0.27	**0.00**	**1.53**	2980K	0.05	0.32
C4OH	1302	0.01	0.27	**0.00**	**0.12**	1041K	0.06	0.46
C6	1302	0.03	0.16	**0.00**	**0.30**	2919K	0.02	0.14
C5OH	1302	0.05	0.29	**0.07**	**0.64**	1418K	0.05	0.31
C8	1302	0.03	0.16	**0.00**	**0.25**	2901K	0.03	0.16
C3DC	1302	0.02	0.16	**0.00**	**0.10**	1176K	0.02	0.13
C10:2	1302	0.01	0.09	**0.00**	**0.50**	1526K	0.01	0.05
C10:1	1302	0.03	0.17	**0.00**	**0.21**	2326K	0.02	0.14
C10	1302	0.04	0.24	**0.00**	**0.35**	2350K	0.03	0.23
C4DC	1302	0.02	0.28	**0.05**	**0.55**	961K	0.08	0.62

C5DC	259	0.01	0.11	**0.00**	**0.37**	1398K	0.02	0.21
C12:1	1302	0.03	0.25	**0.00**	**0.33**	2236K	0.02	0.24
C12	1302	0.06	0.49	**0.00**	**0.59**	2185K	0.03	0.35
C6DC	1302	0.00	0.09	**0.00**	**0.54**	1959K	0.01	0.20
C14:1	1302	0.09	0.50	**0.00**	**0.50**	2944K	0.03	0.32
C14	1302	0.08	1.04	**0.00**	**0.20**	2948K	0.08	0.45
C16:1	1302	0.07	0.40	**0.00**	**0.58**	1831K	0.05	0.41
C16	1302	1.10	6.50	**0.50**	**9.30**	4862K	1.04	5.57
C16OH	1302	0.01	0.07	**0.00**	**0.30**	1476K	0.02	0.10
C18:1	1302	0.42	2.21	**0.00**	**3.00**	2745K	0.53	2.50
C18	1302	0.37	2.37	**0.00**	**2.00**	2294K	0.32	1.71
C18:1 OH	1302	0.01	0.07	**0.00**	**2.00**	2765K	0.01	0.06
C18 OH	1302	0.01	0.04	**0.00**	**2.00**	1616K	0.01	0.05
Alanine	1302	61.11	354.51	**0.0**	**1000.0**	**N/A**	**N/A**	**N/A**
Proline	1302	54.05	428.4	**0.00**	**440.0**	1223K	106	366
Valine	1302	39.69	169.89	**43.0**	**290.0**	2246K	57.7	226
xLeuc	1302	42.32	215.92	**36.0**	**245.0**	2782K	63.2	237
Methionine	1302	6.98	44.05	**6.0**	**63.0**	2961K	11.41	40.3
Phenylalanine	1302	24.27	88.0	**26.0**	**180.0**	2920K	33.4	89.1
Tyrosine	1302	18.95	212.37	**16.0**	**200.0**	4985K	38.6	192.0
Ornithine	1302	35.01	255.2	**0.0**	**300.0**	2370K	20.66	235.0
Citrulline	1302	2.14	28.18	**0.0**	**75.0**	5016K	5.85	26.6
Arginine	1302	2.53	25.28	**0.0**	**132.0**	2679K	1.74	32.9
Glycine	1302	138.8	740.12	**108.0**	**1000.0**	1764K	215.0	803.0
ASA1	1302	0.00	0.01	**0.00**	**0.05**	N/A	N/A	N/A
ASA2	1302	0.00	0.01	**0.00**	**0.05**	N/A	N/A	N/A
C3/C2	1302	0.05	0.33	**0.00**	**0.40**	2751K	0.03	0.19
C4/C2	1302	0.01	0.11	**0.00**	**0.18**	2666K	0.004	0.03
C5/C2	1302	0.01	0.09	**0.00**	**0.16**	2734K	0.002	0.02
C14:1/C12:1	1302	1.38	6.67	**0.00**	**3.00**	2178K	0.67	5.25
C14:1/C16	1302	0.02	0.10	**0.00**	**0.20**	2793K	0.015	0.15
C8/C2	1302	0.0011	0.0312	**N/A**	**N/A**	2662K	0.0011	0.0093
C14:1/C2	1302	0.004	0.104	**N/A**	**N/A**	2725K	0.0016	0.016
C16/C2	1302	0.062	1.249	**N/A**	**N/A**	2693K	0.041	0.28
C8/C10	1302	0.38	1.25	**N/A**	**N/A**	2199K	0.40	1.50
C12/C10	1302	0.90	4.30	**N/A**	**N/A**	2063K	0.57	3.17
C16OH/C16	1302	0.00	0.03	**N/A**	**N/A**	2658K	0.003	0.029
Leuc/Phe	1302	1.23	4.69	**0.0**	**5.0**	2722K	1.12	4.58
Met/Phe	1302	0.19	1.23	**0.0**	**1.0**	2831K	0.21	0.74
Phe/Tyr	1302	0.20	2.61	**0.0**	**3.10**	2781K	0.27	1.40

SQUH: Sultan Qaboos University Hospital, RR: reference range, CLIR: Collaborative Laboratory Integrated Reports, N: number of samples, $P_{1:}$ first percentile, P_{99}: ninety nineth percentile, Bot: lower limit of currently adopted reference range, Top: upper limit of the currently adopted reference range,

Discussion

MS/MS is an effective tool in screening for multiple metabolic disorders in a single analysis and it is utilized increasingly for neonatal screening for IEMs worldwide. Neonatal screening programs for IEMs aim for detection of metabolic disorders early after birth to facilitate appropriate interventions to avoid or ameliorate adverse outcomes. Despite that some metabolic disorders detected by MS/MS maybe untreatable, establishment of a diagnostic etiology is still valuable to families.[16] Although many nations around the globe currently perform newborn screening for IEMs as a public health measure, newborn screening is still not widely applied in many Arab countries. National MS/MS NBS is available in Qatar and Saudi Arabia. Private MS/MS is available in Lebanon,[17] while in Egypt it was available in some universities up until recently.[18] In Oman, selective MS/MS-based high-risk screening for IEMs was introduced at SQUH since 2002, and our previous report shows that different varieties of IEMs are prevalent in the Omani community.[19] Although national NBS is not presently available in Oman, this is anticipated to change in the near future, and the country may soon benefit from a nationwide NBS program for treatable IEMs. Of particular relevance, establishment of reference ranges for ACs

and AAs for Omani population may prove timely useful and clinically valuable when deciding about cut off values that maybe adopted for analytes used in the awaited NBS program in Oman. This is the first study on screening of IEMs using MS/MS in Oman, where the reference range values of individual AAs and ACs were established by analyzing the DBS specimens of healthy subjects.

The disorders detected by most MS/MS newborn screening programs can divided into three main groups: aminoacidopathies, organic acidemias and fatty acids oxidation disorders.[20] MS/MS can distinctly diagnose most of these disorders. However, analytes reference cut-offs limits are required to detect the IEM-related disorders. Reliable cut-offs would help to minimize the false positive or false negative cases.[21-23] Through a worldwide collaborative project (R4S and then CLIR), the cut-offs values for screening of IEMs were determined using a large number of healthy newborns.[23,15]

Herein, we reported neonatal reference ranges for ACs and AAs in DBS using derivatized non-kit MS/MS method conducted on Omani neonates. The old reference ranges which were used at SQUH since 2003 were based on Saudi neonatal population. However, the laboratory holds records of frequent false-positives of myristoylcarnitine (C14) and C18, confirmed to be normal on exclusionary diagnostic testing. On the other hand, sadly a number of affected positive cases were missed partly because of higher cut offs assigned for C3, C4, C5:1, C5OH, C18:1, C18:1OH, C18OH, and Phe. In comparison with 1st and 99th percentiles reported in CLIR for ACs and AAs, we found values in similar ranges for most of them using a much smaller sample size. Our data shows a significant effect of age on the concentration of most of ACs and AAs, which underscore the need to establish an age-specific reference range for these analytes, and to consider age of the patient when interpreting MS/MS screening results to improve their diagnostic value for detection of IEMs in different age groups. On the other hand, although gender had an impact on the abundance of some analytes, it has not affected their discriminatory diagnostic values when considering the reference range.

One of the limitations of this study is the relatively small samples size used for determination of reference ranges. Analysis of a larger sample size maybe required for more reliable reference intervals that maybe generalizable to the Omani population. It is intuitively reassuring though that the values obtained and percentiles distribution has largely been in agreement with the pooled data collected in CLIR. Another potential threat to this study is sampling bias that may have resulted from sampling newborns delivered at SQUH. The university hospital based in the capital, Muscat, may not be representative of the more heterogeneous population outside this governorate. Arguably though, SQUH and SQU staffs and their families typically utilizing maternity and delivery services at SQUH originate from a more heterogeneous demographic, social and geographical area of the country that is not bound by the governorate of Muscat.

Conclusion

In conclusion, MS/MS technique plays an important role in screening and diagnosis of IEMs. The current study is the first to establish reference intervals for AAs and ACs in DBS samples from healthy Omani newborns, and besides its current diagnostic impact, it may prove to be of significant value in the future when considering analytes cut offs for NBS once established in Oman.

Statement of Ethics

This research study was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. The Medical Research Ethics Committee (MREC), College of Medicine and Health Sciences, Sultan Qaboos University approved this study (REF. No. SQU-EC/036/2020 MREC # 2092).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This research required no funding. Analysis was funded through the clinical research and development scheme in the hospital.

Disclosure

The abstract of this manuscript has been published as a poster of presentation through Int. J. Neonatal Screen. 2021, 7(4), 71; https://doi.org/10.3390/ijns7040071, and can be viewed online at https://www.mdpi.com/2409-515X/7/4/71/htm. All authors approved the manuscript and its submission to the Oman Medical Journal, and none of the authors have published or submitted any related papers from the same study.

Author Contributions

Al-Riyami S designed the research, conducted the research, performed statistical analysis, analysed data and wrote the first draft of the manuscript. Al-Maney M, Al-Fahdi A and Al-Maskri S conducted the research and performed MS/MS analysis for AAs and ACs. Al-Thihli K designed the research, analysed data and reviewed the manuscript.

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