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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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ABSTRACT

Objectives: To establish a reference range for acylcarnitines (ACs) and amino acids (AAs) concentrations in dried blood spot (DBS) samples of Omani neonates to detect inborn errors of metabolism (IEM), and to evaluate the effect of age and sex on ACs and AAs. ***Methods:*** 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 between August 2008 and May 2009. ***Results:*** More than fifty biomarkers that allow diagnosis of various IEMs were measured, their 1st and 99th percentile values determined, and compared with published international data. Our results were comparable with the corresponding figures from Collaborative Laboratory Integrated Report, despite a much smaller sample size. We found that age had a significant effect on most ACs and AAs except decadienoylcarnitin, decenoylcarnitine, adipylcarnitine, palmitoylcarnitine, steatoylcarnitine, tyrosin, phenylalanine, and valine. Sex of the neonate had insignificant effect on most ACs and AAs except free-carnitine, acetylcarnitine, hexanoylcarnitine, octanoylcarnitine, malonylcarnitine, decanoylcarnitine, dodecenoylcarnitine, dodecanoylcarnitine, and tetradecanoylcarnitine. ***Conclusions:*** Tandem mass spectrometer is a highly effective tool for high throughput screening of IEM. 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 for newborn screening in the near future.

Inborn errors of metabolisms (IEMs) are a rare group of genetic disorders that can produce serious clinical consequences for an affected neonate or infant.[1] IEMs are inherited biochemical disorders 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 or deficiency in the ultimate products of the pathway.[2] Late diagnosis may result in consequences including 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: lactate, ammonia, amino acids (AAs) in plasma, urinary organic acids, and acylcarnitine (AC) profiles.[3]

Tandem mass spectrometry (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 is being routinely used in many clinical biochemical laboratories worldwide,[6–10] having replaced the traditional single-analyte screening techniques.

Because of the importance of the presence of rigorous reference range (RR) for AAs and ACs in MS/MS, this study is focused on the establishment of concentration values of AAs and ACs in Omani newborn population using derivatized non-kit Electrospray-ionization MS/MS method. The influence of age and sex on ACs and AAs has been documented.[11–14] Therefore, the effect of these two factors on concentrations in dry blood spot (DBS) was evaluated in this study.

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METHODS

The subjects of this study were N = 1302 healthy Omani neonates of both sexes, delivered at Sultan Qaboos University Hospital (SQUH) between August 2008 and May 2009. They were all delivered at term (37–42 weeks gestation), had appropriate weights for their gestational ages (2500–4000 g), and required no active resuscitation at birth, having APGAR scores > 7 at one and five minutes. There was no clinical evidence of congenital anomalies on routine newborn examination. Blood specimens were collected by heel prick 24 hours to seven 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 °C until analysis. The healthy newborns were divided into three groups according to age. Group 1: < 48 hours (n = 1026, 78.8%), group 2: 48–72 hours (n = 171, 13.1%), and group 3: > 72 hours (n = 105, 8.1%).

This study, being a retrospective review, used the previously collected electronic data available in the hospital database, pertaining to an earlier pilot study during which the consent of the caregivers of the neonates had been obtained. No further patient contact or involvement was required. This research project was conducted ethically in accordance with the World Medical Association Declaration of Helsinki and approved by the Medical Research Ethics Committee (MREC), College of Medicine and Health Sciences, Sultan Qaboos University (REF. No. SQU-EC/036/2020 MREC # 2092).

Isotopically labeled internal standards of AAs and ACs were purchased from Cambridge Isotope Laboratories (Andover, USA), 3M butanol HCl was purchased from Regis Technologies (Morton Grove, USA), HPLC-grade acetonitrile and methanol were purchased from Sigma-Aldrich (Steinheim, Germany) and VWR International (Geldenaaksebaan, 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 the Center of Disease Control and Prevention. Dry blood samples were analyzed using Waters ACQUITY UPLC™ system coupled to MS/MS TQD (Micromass UK Limited, Manchester, UK).

A 3.2 mm punch of the DBS was 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 minutes at 300 rpm. The extract was transferred to a new polystyrene 96-well plate and dried on heating block at 45 °C under airflow for approximately 30 minutes. Then, 60 µL of butanol HCl was added and the plate was sealed with adhesive film and incubated at 65 °C for 15 minutes. The mixture was again dried for about 45 minutes. Finally, the residue was reconstituted with 100 µL of mobile phase acetonitrile: water at 80:20 (v/v), covered with aluminum foil, shaken for 5–10 minutes at 300 rpm 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 °C, 250 °C, 65 L/h, and 550 L/h, respectively. The samples were run using an isocratic mobile phase consisting of acetonitrile: water at 80:20 (v/v) and injected directly into the MS/MS via a pipeline connected to the liquid chromatography without chromatographic separation. The flow rate was 300 µL/min and 20 µL of the sample was injected. The total run time was 1.7 min per sample. The analysis was done using six 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); and (6) neutral loss of m/z 102 (scan range: m/z 130–150). The MassLynx™ version 4.1 and NeoLynx software (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.

SPSS windows (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.) was used for statistical analysis and to calculate the 1st, 5th, 10th, 90th, 95th, and 99th percentiles of each AC and AA. Shapiro-Wilk test was used to check the normal

distribution of data. Correlations between age, sex, and each analyte concentration were tested with a Spearman test. A *p*-value of ≤ 0.050 was considered statistically significant.

RESULTS

A total of N = 1302 healthy Omani neonates (674 male, 628 female) aged 1–7 days were screened in this study. They 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 age at collection was 40.0±29.0 hours. Six neonates were tested < 24 hours of life: 18 hours (n = 1), 20 hours (n = 1), 21 hours (n = 3), and 22 hours (n = 1). It was ensured that all the six 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 hydroxyisovalerylcarnitine ACs were the most abundant. Higher concentrations were observed for free-carnitine (C0) (mean = 24.0 µM) and acetylcarnitine (C2) (mean = 16.14 µM), whereas hydroxyoctadecanoylcarnitine (C18-OH) was the least abundant AC with mean concentration of 0.02 µM. Hexadecanoylcarnitine (C16) was the most abundant among all long-chain ACs 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 correlations with age and sex. All the data sets showed normal distribution when evaluated by the Shapiro-Wilk test. Therefore, a Pearson correlation test was used to check the behavior of various concentrations of ACs and AAs with respect to the two variables age and sex. All analytes were found to be significantly affected by age except for decenoylcarnitine (C10:1), decadienoylcarnitine (C10:2), adipylcarnitine (C6DC), palmitoylcarnitine (C16:1), steatoylcarnitine (C18), tyrosine (Tyr), phenylalanine (Phe), and valine (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

Table 1: Reference range from a current study (new) compared with what was used in SQUH (old) for individual acylcarnitines and amino acids with selected commonly used ratios in dried blood spots of healthy neonates using tandem mass spectrometer.

Marker or ratio	New SQUH RR			Old SQUH RR		CLIR RR		
	N	P_1	P_{99}	Bot	Top	N	P_1	P_{99}
Free-carnitine (C0)	1302	10.34	56.90	6.00	72.00	2924K	9.73	49.00
Acetylcarnitine (C2)	1302	1.97	52.10	0.00	74.00	2798K	8.42	47.60
Propionylcarnitine (C3)	1302	0.58	6.09	0.00	10.00	5028K	0.60	4.01
Butyrylcarnitine (C4)	1302	0.10	0.92	0.00	1.80	2882K	0.10	0.57
Tiglylcarnitine (C5:1)	1302	0.01	0.09	0.00	0.50	1726K	0.01	0.06
Isovalerylcarnitine (C5)	1302	0.05	0.27	0.00	1.53	2980K	0.05	0.32
Hydroxybutyrylcarnitine (C4OH)	1302	0.01	0.27	0.00	0.12	1041K	0.06	0.46
Hexanoylcarnitine (C6)	1302	0.03	0.16	0.00	0.30	2919K	0.02	0.14
Hydroxyisovalerylcarnitine (C5OH)	1302	0.05	0.29	0.07	0.64	1418K	0.05	0.31
Octanoylcarnitine (C8)	1302	0.03	0.16	0.00	0.25	2901K	0.03	0.16
Malonylcarnitine (C3DC)	1302	0.02	0.16	0.00	0.10	1176K	0.02	0.13
Decadienoylcarnitine (C10:2)	1302	0.01	0.09	0.00	0.50	1526K	0.01	0.05
Decenoylcarnitine (C10:1)	1302	0.03	0.17	0.00	0.21	2326K	0.02	0.14

Table 1: Reference range from a current study (new) compared what was in used in SQUH (old) for individual acylcarnitines and amino acids with selected commonly used ratios in dried blood spots of healthy neonates using tandem mass spectrometer.

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Marker or ratio	New SQUH RR			Old SQUH RR		CLIR RR		
	N	P_1	P_{99}	Bot	Top	N	P_1	P_{99}
Decanoylcarnitine (C10)	1302	0.04	0.24	0.00	0.35	2350K	0.03	0.23
Methylmalonylcarnitine (C4DC)	1302	0.02	0.28	0.05	0.55	961K	0.08	0.62
Glutarylcarnitine (C5DC)	259	0.01	0.11	0.00	0.37	1398K	0.02	0.21
Dodecenoylcarnitine (C12:1)	1302	0.03	0.25	0.00	0.33	2236K	0.02	0.24
Dodecanoylcarntine (C12)	1302	0.06	0.49	0.00	0.59	2185K	0.03	0.35
Adipylcarnitine (C6DC)	1302	0.00	0.09	0.00	0.54	1959K	0.01	0.20
Tetradecanoylcarnitine (C14:1)	1302	0.09	0.50	0.00	0.50	2944K	0.03	0.32
Myristoylcarnitine (C14)	1302	0.08	1.04	0.00	0.20	2948K	0.08	0.45
Palmitoylcarnitine (C16:1)	1302	0.07	0.40	0.00	0.58	1831K	0.05	0.41
Hexadecanoylcarnitine (C16)	1302	1.10	6.50	0.50	9.30	4862K	1.04	5.57
Hydroxyhexadecanoylcarnitine (C16OH)	1302	0.01	0.07	0.00	0.30	1476K	0.02	0.10
Octadecenoylcarnitine (C18:1)	1302	0.42	2.21	0.00	3.00	2745K	0.53	2.50
Steatoylcarnitine (C18)	1302	0.37	2.37	0.00	2.00	2294K	0.32	1.71
Hydroxyoctadecenoylcarnitine (C18:1 OH)	1302	0.01	0.07	0.00	2.00	2765K	0.01	0.06
Hydroxyoctadecanoylcarnitine (C18 OH)	1302	0.01	0.04	0.00	2.00	1616K	0.01	0.05
Alanine	1302	61.11	354.51	0.00	1000.00	N/A	N/A	N/A
Proline	1302	54.05	428.40	0.00	440.00	1223K	106.00	366.00
Valine	1302	39.69	169.89	43.0	290.00	2246K	57.70	226.00
xLeuc	1302	42.32	215.92	36.0	245.00	2782K	63.20	237.00
Methionine	1302	6.98	44.05	6.00	63.00	2961K	11.41	40.30
Phenylalanine	1302	24.27	88.00	26.00	180.00	2920K	33.40	89.10
Tyrosine	1302	18.95	212.37	16.00	200.00	4985K	38.60	192.00
Ornithine	1302	35.01	255.2	0.00	300.00	2370K	20.66	235.00
Citrulline	1302	2.14	28.18	0.00	75.00	5016K	5.85	26.60
Arginine	1302	2.53	25.28	0.00	132.00	2679K	1.74	32.90
Glycine	1302	138.80	740.12	108.00	1000.00	1764K	215.00	803.00
Argininosuccinic acid-1 (ASA1)	1302	0.00	0.01	0.00	0.05	N/A	N/A	N/A
Argininosuccinic acid-2 (ASA2)	1302	0.00	0.01	0.00	0.05	N/A	N/A	N/A
propionylcarnitine (C3)/ acetylcarnitine (C2)	1302	0.05	0.33	0.00	0.40	2751K	0.03	0.19
butyrylcarnitine (C4)/ acetylcarnitine (C2)	1302	0.01	0.11	0.00	0.18	2666K	0.00	0.03
isovalerylcarnitine (C5)/ acetylcarnitine (C2)	1302	0.01	0.09	0.00	0.16	2734K	0.00	0.02
Tetradecanoylcarnitine (C14:1)/dodecenoylcarnitine (C12:1)	1302	1.38	6.67	0.00	3.00	2178K	0.67	5.25
Tetradecanoylcarnitine (C14:1)/ hexadecanoylcarnitine (C16)	1302	0.02	0.10	0.00	0.20	2793K	0.015	0.15
Octanoylcarnitine (C8)/ acetylcarnitine (C2)	1302	0.00	0.03	N/A	N/A	2662K	0.00	0.01

Table 1: Reference range from a current study (new) compared with what was used in SQUH (old) for individual acylcarnitines and amino acids with selected commonly used ratios in dried blood spots of healthy neonates using tandem mass spectrometer.

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Marker or ratio	New SQUH RR			Old SQUH RR		CLIR RR		
	N	P_1	P_{99}	Bot	Top	N	P_1	P_{99}
Tetradecanoylcarnitine (C14:1)/ Acetylcarnitine (C2)	1302	0.00	0.10	N/A	N/A	2725K	0.00	0.02
Hexadecanoylcarnitine (C16)/ Acetylcarnitine (C2)	1302	0.06	1.25	N/A	N/A	2693K	0.04	0.28
Octanoylcarnitine (C8)/ Decanoylcarnitine (C10)	1302	0.38	1.25	N/A	N/A	2199K	0.40	1.50
Dodecanoylcarntine (C12)/ Decanoylcarnitine (C10)	1302	0.90	4.30	N/A	N/A	2063K	0.57	3.17
Hydroxyhexadecanoylcarnitine (C16OH)/ Hexadecanoylcarnitine (C16)	1302	0.00	0.03	N/A	N/A	2658K	0.00	0.03
Leuc/Phe	1302	1.23	4.69	0.00	5.00	2722K	1.12	4.58
Met/Phe	1302	0.19	1.23	0.00	1.00	2831K	0.21	0.74
Phe/Tyr	1302	0.20	2.61	0.00	3.10	2781K	0.27	1.40

New: new references range generated by the current study; Old: reference range was adopted from a Saudi study; SQUH: Sultan Qaboos University Hospital; RR: reference range; CLIR: Collaborative Laboratory Integrated Reports; N: number of samples; P1: first percentile; P99: 99th percentile; Bot: lower limit of currently adopted reference range; Top: upper limit of the currently adopted reference range; K: (Thousand); N/A: not available.

(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), hexanoylcarnitine (C6), octanoylcarnitine (C8), C10, dodecenoylcarnitine (C12:1), dodecanoylcarnitine (C12), tetradecanoylcarnitine (C14:1), C16, C18, and C18:1-OH. The sex of the neonate had a significant effect on C0, C2, C6, C8, C3DC, C10, C12:1, C12, and C14:1. In all analytes that were significantly correlated with sex, concentrations were higher in males than in females.

Our ACs and AAs' 1st, 10th, 50th, 90th, and 99th percentile values were compared with Collaborative Laboratory Integrated Reports (CLIR) RR,[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 that of CLIR, the 99th percentile values for ACs and AAs are comparable, and are substantially different from the currently adopted RRs in our laboratory, which were based on newborn screening (NBS) study carried out in Saudi Arabia in which 5000 healthy neonates were screened and the RRs assigned as 0.5% (bottom) and 99.5% (top) [Table 1].

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. Even though some metabolic disorders detected by MS/MS maybe untreatable, establishment of a diagnostic etiology is still valuable to families, given genetic counseling implications.[16] Although many nations around the globe currently perform NBS for IEMs as a public health measure, this is still not widely applied in many Arab countries. National MS/MS NBS is available in Qatar and Saudi Arabia and private MS/MS is available in Lebanon,[17] In Egypt, it was available in some universities until recently.[18] In Oman, selective MS/MS-based high-risk screening for IEMs was introduced at SQUH in 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, the country may soon benefit from a nationwide NBS program for treatable IEMs. Of particular relevance, establishment of RRs for ACs and AAs for the 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 the screening of IEMs using MS/MS in Oman, where the RR values of individual AAs and ACs were established by analyzing the DBS specimens of healthy subjects.

The disorders detected by most MS/MS NBS programs fall into three main categories: aminoacidopathies, organic acidemias, and fatty acids oxidation disorders.[20] MS/MS can distinctly diagnose most of these. However, analytes reference cut-off limits are required to detect 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-off values for screening of IEMs have been determined using a large number of healthy newborns.[15,23]

The current study has generated for the first time in Oman, a set of neonatal RRs for ACs and AAs in DBS using derivatized non-kit MS/MS method conducted on Omani neonates. This has replaced the old RRs based on the Saudi neonatal population that were being used at SQUH since 2003. This had resulted in frequent false positives of myristoylcarnitine and C18, which were later confirmed to be normal on exclusionary diagnostic testing. More problematic were the many positive cases that were missed partly because of the 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 RR 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. The impact of the sex of the infant was much less significant and had an impact on the abundance of some analytes, it has not affected their discriminatory diagnostic values when considering the RR.

Among the limitations of this study is the relatively small sample size used to determine the RRs. A larger study with a heterogeneous cohort will generate a more reliable set of reference intervals more generalizable to the Omani population. However, it is intuitively reassuring that even with this small sample, the values obtained, and the distribution of percentiles are largely in agreement with the internationally pooled data collected in CLIR. Another limitation of our study is its urban sampling bias, SQUH being located in the highly urbanized Muscat governorate situated in the north-eastern coastal strip of this geographically vast country. Arguably though, SQUH and SQU staff and their families, who typically utilize the maternity and delivery services at SQUH, originate from different geographical areas of the country, often outside the governorate of Muscat.

CONCLUSION

MS/MS technique plays a vital role in the 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, our results may prove to be of significant value in the future when considering analytes cut-offs for NBS once established in Oman.

Disclosure

The authors declared no conflicts of interest. No funding was received for this study. However,analysis was funded through the clinical research and development scheme in the hospital. The abstract of this paper 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.

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