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Determination of Normal Range of Acylcarnitine in Neonatal Dried Blood Spots using LC-MS/MS

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Abstract

Background: Acylcarnitine is one of the crucial markers of fatty acid metabolism, and examination of their level in infants can reveal several Inherited Metabolic Disorders (IDM) or Inborn errors of Metabolism (IEM). Because of the great importance of hereditary, metabolic, and other inherited disorders early diagnosis before the appearance of clinical symptoms, this study was carried out to establish a reference range for carnitine analytes and to identify acylcarnitine profiles in normal weight neonatal dried blood spots (DBS) specimens.

Methods: By using liquid chromatography tandem mass spectrometry (LC-MS/MS) for neonatal screening and eventually the examination and analysis of LC-MS/MS results, 34 acylcarnitine derivatives were identified.

Results: The normal range for acylcarnitine analytes with carbon numbers ranging from zero to 18, both main and the branched ones, were ultimately measured. Afterward, they were compared with the results of some other diagnostic laboratories to be verified.

Conclusion: This study differed from the other findings, which could be due to diversity in population and work methods**.** However, the reference range of most acylcarnitine derivatives in Tehran closely aligned with this study's findings.

Keywords: Acylcarnitines, Dried Blood Spot, LC-MS/MS, Newborn screening, Reference range.

Introduction

IDMs are a type of rare congenital disease resulting from genetic defects in enzymes that are engaged in metabolism pathways and eventually lead to oscillations in the amount of acylcarnitines as well as amino acids (1, 2). Acylcarnitines are one of the important elements of fatty acid metabolism, and examination of their level in infants could be a diagnostic method for several IDMs. Many significances are considered for L-carnitine in mammals, such as the survival of mammalians through L-carnitine's pivotal role in facilitating the oxidation of mitochondrial and peroxisomal fatty acids as well as in modulating the intracellular relationship between free CoA and acyl-CoA which result in maintaining the concentrations of L-carnitine and its ester derivatives in endogenous plasma and other tissues (3, 4). Every alteration in the concentration of carnitine in plasma or tissue, either less than the necessary amount for normal functioning of the organism or more than it, leads to carnitine deficiency. According to clinical data, tissue carnitine content less than 10-20% of the standard measures for the physiological effect should be

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considered clinically significant. Decreased levels of free carnitine often occur in infants born to mothers with carnitine deficiency as well as metabolic disorders (5). There are several types of carnitine deficiency, all of which eventually lead to the impaired entry of fatty acids into the mitochondria, resulting in defective lipid oxidation. Primary systemic carnitine deficiency (SCD) is characterized by reduced carnitine concentrations in both plasma and damaged tissues, including the heart, skeletal muscle, and liver which usually reaches under 10% of normal values of those, and defects in this process are due to abnormalities associated with carnitine uptake into cells or impaired renal carnitine transport (6). The initial onset of systemic carnitine deficiency is from the first month of life to 7 years with an average of two years, which has different types of clinical symptoms such as progressive cardiomyopathy, acute encephalopathy with hypoglycemic hypoglycemia, and myopathy. Secondary carnitine deficiency, which results from decreased plasma or tissue carnitine, may also be associated with a wide range of genetic diseases, Iatrogenic agents, repeated dialysis treatment for a long time, and treatment with certain drugs such as valproate and pivampicillin. Carnitine deficiency is expected to cause a variety of disorders in the function of several organs, including the liver, skeletal muscle, heart, and brain (7-9). Measurement of acylcarnitine in the blood is a valuable test for biochemical screening and diagnosis of inherited disorders of mitochondrial fatty acid oxidation. Though, little is known about the different reference ranges of acylcarnitines and whether these reference domains are age- or sex-dependent (10, 11). Measurements of acylcarnitine species are often used to assess symptomatic patients, neonatal screening and follow-up testing, prenatal diagnosis, and post-mortem screening (12). Numerous analytical methods such as spectrophotometry, chromatography, capillary electrophoresis, and radio enzymatic are used to determine acylcarnitines in neonatal screening programs. However, LC-MS/MS has been the technique used primarily for IDM diagnosis and screening in recent investigations due to its power and sensitivity in detecting Acylcarnitines and amino acids (13). This method can detect many disorders related to acylcarnitine, such as propionic acid (PA), Methylmalonic acid (MMA), Medium Chain Acyl CoA Dehydrogenase Deficiency (MCAD), Very Long Chain Acyl CoA Dehydrogenase Deficiency (VLCAD), long-chain hydroxy acyl CoA dehydrogenase (LCHAD) deficiency, citrullinemia, and argininosuccinic aciduria which gives the diagnosis. The frequency of each of these type of disorders in different ethnic groups can be diverse. Mass Spectroscopy (MS) has the ability to detect and screen many inherited diseases of metabolism intermediates in symptomatic infants. DBS analysis of acylcarnitine by sequential mass spectrometry has high specificity and sensitivity for diagnosing the disease in infancy. Nevertheless, this process does not differentiate isobaric compounds and causes certain interferences due to the presence of acylcarnitines with the same mass as well as several exogenous disturbing sources; hence using the LC-MS/MS method covers the limitations and disadvantages and leads to more accurate and reliable measurement and results (12, 14). The aim of this study was to establish a reference range for carnitine analytes and to identify acylcarnitine profiles in normal-weight neonatal DBS specimens by applying LC-MS/MS for neonatal screening to diagnose hereditary, metabolic, and other inherited disorders before the manifestation of clinical signs and symptoms.

Materials and Methods

Samples collection

This study included approximately 1000 samples of dried blood from children 3 to 7 days (boys and girls)**.** All samples were prepared from the North, Razavi, and South Khorasan provinces. After preparation, all of them were referred to our laboratory for analysis of acylcarnitines and their results**.** All steps were performed in accordance with the

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ethical standards of the Human Experiments Committee of MUMS (Mashhad University of Medical Sciences).

Chemicals, reagents, and tools

In this study, commercial chemical reagents A, B, and C, with the highest purity, were purchased from the German company RECIPE. The combination was also used as a derivative reagent. The LC-MS/MS model API 3200 was also applied to analyze the acylcarnitines profile.

Extraction, derivation, and liquid chromatography-mass spectrometry

Acylcarnitines in dried blood samples were extracted using organic solvent and then identified and quantified by LC-MS/MS**.** For this purpose, firstly, 3 ml of reagent A was added to Internal standard acylcarnitine powder, and mix occasionally and gently for about 15 min. Finally fill the volumetric flask with reagent A exactly to 100 ml and stir for homogeneity and stored in the refrigerator. Then 100 µl of prepared reagent A was added to vials containing punched dried blood samples and placed on a shaker for 30 minutes. After 30 minutes of shaking, the solutions were transferred to new vials and kept under evaporation (at 40 °C) for 20 minutes**.** Secondly, 50 µl of reagent B was added to the vials evaporated previously, placed on a shaker at 60 °C for 5 minutes, and then kept under the evaporator for 20 minutes to be dried again. Thirdly, 100 µl of reagent C was inoculated into the vials and placed on a shaker for 5 minutes at 25 °C**.** These vials were afterward transferred to 96-well plates with negative control samples. Finally, the samples were placed in the LC-MS/MS device and were analyzed.

Statistical analysis

Statistical analysis was performed using SPSS version 16 and Excel. The identified compounds and their concentration range were determined for all individuals.

Results

Determination of acylcarnitine derivatives

In this study, more than 1000 samples of dried blood of children aged 3 to 7 days were provided from the North, Razavi, and South Khorasan provinces. The frequency and concentration range of each acylcarnitine derivative were assessed by applying LC-MS/MS analysis. Amongst the examined samples, 34 acylcarnitine derivatives, including Free carnitine, Acetylcarnitine, Propionylcarnitine, Malonylcarnitine, Butyrylcarnitine, Hydroxybutyrylcarnitine, Methylmalonylcarnitine, Isovalerylcarnitine, Tiglylcarnitine, Hydroxyisovalerylcarnitine, Glutarylcarnitine, Hexanoylcarnitine, Adipoylcarnitine, Octanoylcarnitine, Octenoylcarnitine, Decanoylcarnitine, Decadienoylcarnitine, Decenoylcarnitine, Dodecanoylcarnitine, Dodecenoylcarnitine, Tetradecanoylcarnitine, Tetradecenoylcarnitine, Tetradecadienoylcarnitine, Hydroxytetradecanoylcarnitine, Hexadecanoylcarnitine, Hexadecenoylcarnitine, Hedroxyhexadecenoylcarnitine, Hydroxyhexadecanoylcarnitine, Octadecanoylcarnitine, Octadecadienoylcarnitine, Octadecenoylcarnitine, Hydroxylinoleoylcarnitine, Hydroxyoleoylcarnitine, and Hydroxystearoylcarnitine were identified. The determined acylcarnitine profile is given in Table 1.

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Table 1. Reference range of 34 items of acylcarnitine, identified using LC-MS/MS.

Analyte	Analyte Full name	Male		Female	
		Lower	Upper	Lower	Upper
C0	Free carnitine	11.854	53.650	12.100	50.664
C2	Acetylcarnitine	12.927	45.255	12.818	48.366
C3	Propionylcarnitine	0.607	3.753	0.583	3.778
C3DC	Malonylcarnitine	0.018	0.066	0.017	0.061
C4	Butyrylcarnitine	0.088	0.553	0.097	0.618
C4OH	Hydroxybutyrylcarnitine	0.045	0.243	0.039	0.245
C4DC	Methylmalonylcarnitine	0.051	0.277	0.071	0.300
C5	Isovalerylcarnitine	0.057	0.266	0.057	0.330
C5:1	Tiglylcarnitine	0.002	0.047	0.002	0.019
C5OH	Hydroxyisovalerylcarnitine	0.060	0.201	0.055	0.199
C5DC	Glutarylcarnitine	0.036	0.147	0.034	0.154
C6	Hexanoylcarnitine	0.018	0.117	0.020	0.082
C6DC	Adipoylcarnitine	0.007	0.046	0.008	0.054
C8	Octanoylcarnitine	0.018	0.081	0.016	0.079
C8:1	Octenoylcarnitine	0.030	0.172	0.027	0.209
C10	Decanoylcarnitine	0.033	0.140	0.022	0.133
C10:2	Decadienoylcarnitine	0.003	0.041	0.003	0.030
C10:1	Decenoylcarnitine	0.002	0.136	0.021	0.113
C12	Dodecanoylcarnitine	0.034	0.138	0.033	0.140
C12:1	Dodecenoylcarnitine	0.078	0.158	0.070	0.144
C14	Tetradecanoylcarnitine	0.071	0.294	0.056	0.295
C14:1	Tetradecenoylcarnitine	0.016	0.118	0.016	0.105
C14:2	Tetradecadienoylcarnitine	0.009	0.035	0.006	0.031
C14OH	Hydroxytetradecanoylcarnitine	0.005	0.032	0.004	0.026
C16	Hexadecanoylcarnitine	0.898	5.116	0.934	5.442
C16:1	Hexadecenoylcarnitine	0.039	0.289	0.039	0.343
C16:1OH	Hedroxyhexadecenoylcarnitine	0.016	0.109	0.016	0.110
C16OH	Hydroxyhexadecanoylcarnitine	0.008	0.043	0.008	0.042
C18	Octadecanoylcarnitine	0.308	1.570	0.364	1.569
C18:2	Octadecadienoylcarnitine	0.097	0.865	0.079	1.067
C18:1	Octadecenoylcarnitine	0.597	3.184	0.665	2.715
C18:2OH	Hydroxylinoleoylcarnitine	0.000	0.039	0.000	0.036
C18:1OH	Hydroxyoleoylcarnitine	0.000	0.055	0.003	0.053
C18OH	Hydroxystearoylcarnitine	0.000	0.028	0.000	0.028

Discussion

For the purpose of verifying our results, the upper and lower concentration cut-offs of acylcarnitine derivatives were randomly selected from 1000 specimens, 520 samples, among which 320 females and 200 males were compared with the findings of ARCHIEMDlife (Austria), University of Hamburg (Germany), Tehran, and North of Iran. Our results were also compared to other studies that were done at Institute of Child Care and Pediatrics (Martagão, Gesteira), Malaria Vaccine and Drug Development Center (Cali, Colombia), and Royal Manchester Children Hospital (Manchester, United Kingdom). Our results showed the lower limits of several derivatives, including

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Acylcarnitine, Butyrylcarnitine, Methylmalonylcarnitine, Hexanoylcarnitine, Adipoylcarnitine, Decenoylcarnitine, Hexadecanoylcarnitine, Octadecanoylcarnitine, Octadecenoylcarnitine, and Hydroxyoleoylcarnitine were higher in females than of those in males.

On the other hand, the upper limit of other acylcarnitine derivatives, such as Free carnitine, Malonylcarnitine, Tiglylcarnitine, Hydroxyisovalerylcarnitine, Hexanoylcarnitine, Octanoylcarnitine, Decanoylcarnitine, Decadienoylcarnitine, Decenoylcarnitine, Dodecenoylcarnitine, Tetradecenoylcarnitine, Tetradecadienoylcarnitine, Hydroxytetradecanoylcarnitine, Hydroxyhexadecanoylcarnitine, Octadecanoylcarnitine, Octadecenoylcarnitine, Hydroxylinoleoylcarnitine, and Hydroxyoleoylcarnitine were higher in males compared to the females.

Interestingly, the lower values of Isovalerylcarnitine, Tiglylcarnitine, Decadienoylcarnitine, Tetradecenoylcarnitine, Hexadecenoylcarnitine, Hydroxyhexadecanoylcarnitine, and Hedroxyhexadecenoylcarnitine were equal in both genders.

Determining the concentration range of acylcarnitine derivatives

After normalizing the data, each acylcarnitine derivative's upper and lower limits were calculated using the following equations (15). The median, mean, standard deviation (SD), and coefficient of variation (CV) were determined for each analyte (15). The results of our studies showed a diverse range of compounds. The following findings were obtained by comparing the reference range of acylcarnitine derivatives in the present study with the results obtained in studies of laboratories mentioned above.

Regarding the reference range of Free carnitine, Propionylcarnitine, Butyrylcarnitine, Malonylcarnitine, Hydroxybutyrylcarnitine, Decenoylcarnitine, Hedroxyhexadecenoylcarnitine, Octadecenoylcarnitine, Hydroxystearoylcarnitine, the results of Germany and Austria were closely similar to the findings of this study.

In contrast, concerning the reference range for Methylmalonylcarnitine, Isovalerylcarnitine, Tiglylcarnitine, Hydroxyisovalerylcarnitine, Glutarylcarnitine, Hexanoylcarnitine, Carnitine, Octanoylcarnitine, Octenoylcarnitine, Decanoylcarnitine, Decadienoylcarnitine, Dodecanoylcarnitine, Dodecenoylcarnitine, Tetradecanoylcarnitine, Tetradecenoylcarnitine, Tetradecadienoylcarnitine, Hydroxytetradecanoylcarnitine, Hexadecanoylcarnitine, Hexadecenoylcarnitine, Hydroxyhexadecanoylcarnitine, Octadecanoylcarnitine, Octadecadienoylcarnitine, Hydroxylinoleoylcarnitine, Hydroxyoleoylcarnitine, the results of Germany and Austria were higher in comparison to our data. In addition, in the study of laboratory in the North of Iran, results of the reference range of some analytes (Acylcarnitine, Acetylcarnitine, Malonylcarnitine, Hydroxybutyrylcarnitine Hydroxyisovalerylcarnitine, Glutarylcarnitine, Adipoylcarnitine, Octenoylcarnitine, Decenoylcarnitine, Dodecenoylcarnitine) were higher than our findings.

There were differences between Germany, Austria, and North of Iran findings and our results; which could be due to differences in population distribution or methods of the investigation used (15, 16), storage, and transportation condition (11). Derivatizing and un-derivatizing would definitely affect the results of Acylcarnitines measures. The technique mostly used for newborn screening worldwide is derivatized LC-MS/MS. Despite possible Acylcarnitine assessment by un-derivatized technique, it has the drawback of less sensitivity to Acylcarnitines that are dicarboxylic and of not distinguishing several

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isobaric Acylcarnitines (13,17). It could obviously be seen that the reference range of most acylcarnitine derivatives in Tehran showed significant similarity to our findings.

Table 2. Comparison of upper and lower limits of acylcarnitine derivatives between boys and girls.

Reference Range		Mashhad		Austria		Tehran		Germany		North of Iran	
Analyte	**Analyte Full name**	**Lower**	**Upper**	**Lower**	**Upper**	**lower**	**upper**	**Lower**	**Upper**	**Lower**	**Upper**
C0	Free carnitine	11.688	54.742	6	100	8	40	10	70	5.85	37.75
C2	Acetylcarnitine	11.200	48.606	1.34	48.81	0	7	10	50	5.78	32.33
C3	Propionylcarnitine	0.557	3.840	0.13	6.6	0.3	4.6	0.5	6	0.32	3.39
C3DC	Malonylcarnitine	0.015	0.069	0	0.5	0	0.05	0	0.3	0.01	0.16
C4	Butyrylcarnitine	0.088	0.591	0.03	0.9	0	0.55	0.09	0.8	0.06	0.51
C4OH	Hydroxybutyrylcarnitine	0.038	0.247	0.01	0.39	0	0.3			0.01	0.16
C4DC	Methylmalonylcarnitine	0.056	0.288			0	0.25	0.08	1.5	0.05	0.58
C5	Isovalerylcarnitine	0.052	0.291	0.02	2	0	0.36	0.02	0.4	0.04	0.33
C5:1	Tiglylcarnitine	0.002	0.023	0	0.2	0	0.03	0	0.25	0.001	0.1
C5OH	Hydroxyisovalerylcarnitine	0.051	0.211	0.02	0.57	0	0.27	0.08	0.47	0.05	0.58
C5DC	Glutarylcarnitine	0.029	0.154	0	0.2	0	0.15	0.01	0.4	0.06	0.52
C6	hexanoylcarnitine	0.017	0.097	0.01	0.13	0	0.09	0	0.18	0.01	0.09
C6DC	Adipoylcarnitine	0.009	0.048			0	0.05	0	0.12	0.07	0.36
C8	Octanoylcarnitine	0.016	0.089	0.01	0.3	0	0.08	0.01	0.4	0.01	0.09
C8:1	Octenoylcarnitine	0.029	0.204			0	0.18	0	0.3	0.02	0.12
C10	Decanoylcarnitine	0.019	0.134	0.01	0.36	0	0.14	0	0.34	0.01	0.11
C10:2	Decadienoylcarnitine	0.003	0.032	0	0.1	0	0.03	0	0.1	0.001	0.1
C10:1	Decenoylcarnitine	0.020	0.127	0	0.3	0	0.12	0	0.13	0.01	0.08
C12	Dodecanoylcarnitine	0.030	0.154	0.01	0.6	0	0.3	0.02	0.3	0.02	0.15
C12:1	Dodecenoylcarnitine	0.072	0.170			0	0.2	0	0.3	0.01	0.08
C14	Tetradecanoylcarnitine	0.054	0.301	0.01	0.57	0	0.35	0.5	0.55	0.04	0.33
C14:1	Tetradecenoylcarnitine	0.015	0.120	0.01	0.38	0	0.05	0.01	0.35	0.01	0.11
C14:2	Tetradecadienoylcarnitine	0.007	0.034			0	0.05	0	0.1	0.001	0.02
C14OH	Hydroxytetradecanoylcarnitine	0.003	0.032			0	0.03	0	0.1	0.001	0.02
C16	Hexadecanoylcarnitine	0.607	5.493	0.62	7.81	0.55	7.08	0.5	8	0.89	5.77
C16:1	Hexadecenoylcarnitine	0.030	0.347			0	0.47	0.02	0.5	0.02	0.29
C16:1OH	Hedroxyhexadecenoylcarnitine	0.015	0.110			0	0.14	0	0.2	0.01	0.07
C16OH	Hydroxyhexadecanoylcarnitine	0.007	0.043	0	0.1	0	0.05	0	0.09	0.001	0.02
C18	Octadecanoylcarnitine	0.259	1.613	0.3	2.4	0.22	1.67	0.3	2.05	0.22	1.95
C18:2	Octadecadienoylcarnitine	0.094	1.030			0.07	0.68	0.02	0.6	0.05	0.57
C18:1	Octadecenoylcarnitine	0.484	2.930	0.06	3.86	0.35	2.5	0.5	3	0.34	2.69
C18:2OH	Hydroxylinoleoylcarnitine	0.000	0.040			0	0.09	0	0.08	0.001	0.03
C18:1OH	Hydroxyoleoylcarnitine	0.000	0.055			0	0.04	0.02	0.6	0.001	0.03
C18OH	Hydroxystearoylcarnitine	0.000	0.032	0	0.09	0	0.03	0	0.1	0.001	0.02

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Ethics statement
All procedures followed were in accordance with the ethical standards of the committee on human experimentation of MUMS (Mashhad University of Medical Science). Informed consent was obtained from all subjects for being included in the study. Upon reviewing, the Ethical Committee approved the above-mentioned protocol in its session hold on October 27, 2021 with code: 4000294. The methodological aspect of the research was in agreement with ethical principles.

Conflicts of interest
All authors declare they have no conflicts of interest.

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