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Tandem Mass Spectrometric Analysis for Disorders in Amino, Organic and Fatty Acid Metabolism : 2 Years of SCL Experience in Korea

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= Abstract =

Background : The SCL began screening of newborns and high risk group blood spots with tandem mass spectrometry (MS/MS) in April 2001. Our goal was to determine approximate prevalence of metabolic disorders, optimization of decision criteria for estimation of preventive effect with early diagnosis. This report describes the ongoing effort to identify more than 30 metabolic disorders by MS/MS in South Korea.

Methods : Blood spot was collected from day 2 to 30 (mostly from day 2 to 10) after birth for newborn. Blood spot of high risk group was from the pediatric patients in NICU, developmental delay, mental retardation, strong family history of metabolic disorders. One punch (3.2 mm ID) of dried blood spots was extracted with 150 μL of methanol containing isotopically labelled amino acids (AA) and acylcarnitines (AC) internal standards. Butanolic HCl was added and incubated at 65℃ for 15 min. The butylated extract was introduced into the inlet of MS/MS. Neutral loss of m/z 102 and parent ion mode of m/z 85 were set for the analyses of AA and AC, respectively. Diagnosis was confirmed by repeating acylcarnitine profile, urine organic acid and plasma amino acid analysis, direct enzyme assay, or molecular testing.

Results : Approximately 31,000 neonates and children were screened and the estimated prevalence (newborn/high risk group), sensitivity, specificity and recall rate amounted to 1 : 2384/1 : 2066, 96.55%, 99.98%, and 0.73%, respectively. Confirmed 28 (0.09%) multiple metabolic disorders (newborn/high risk) were as follows; 13 amino acid disorders [classical PKU (3/4), BH4 deficient-hyperphenylalaninemia (0/1), Citrullinemia (1/0), Homocystinuria (0/2), Hypermethioninemia (0/1), Tyrosinemia (1/0)], 8 organic acidurias [Propionic aciduria (2/1), Methylmalonic aciduria (0/1), Isovaleric aciduria (1/1), 3-methylcrotonylglycineuria (1/0), Glutaric aciduria typel (1/0)], 7 fatty acid oxidation disorders [LCHAD def. (2/2), Mitochondrial TFP def. (0/1), VLCAD def. (1/0), LC3KT def. (0/1).

Conclusion : The relatively normal development of 10 patients with metabolic disorders among newborns (except for the expired) demonstrates the usefulness of newborn screening by MS/MS for early diagnosis and medical intervention. However, close coordination between the MS/MS screening laboratory and the metabolic clinic/biochmical geneticists is needed to determine proper decision of screening parameters, confirmation diagnosis, follow-up scheme and additional tests.

Key Words : Tandem mass spectrometry, Screening, Metabolic disorder

Introduction

Newborn screening (NBS) for selected inherited metabolic disorders is well-established and now more than 10 millions of newborns are screened a year world wide especially in case for Phenylketonuria (PKU), and Congenital hypothyroidism (CH). There are assay methods such as enzyme immune assay and radio immune assay for metabolic disorder screening of PKU and CH, nonetheless the most powerful tool ever used for the screening of multiple metabolic disorders is the newborn screening by tandem mass spectrometry (MS/MS). Quantitative analysis of amino acids (AA) and acylcarnitines (AC) using MS/MS is an emerging technology used to neonatal dried blood spot specimen. Numerous papers described the utility of MS/MS for amino, organic, fatty acid, and very long chain fatty acid or combinations of organic, amino and fatty acid metabolic disorders etc. Early diagnosis and treatment can reduce the morbidity, mortality and even social cost associated with these diseases. MS/MS newborn screening has critical clinical significance to provide substantial benefit to newborns for favorable treatment and outcome if diagnosed early in life.

Before March 1997, no newborn screening program has screened >650,000 Korean newborns annually for two mandated disorders. The two mandated disorders were PKU and CH. The first pioneering onset of newborn screening was conducted at the department of pediatrics, Soonchunhyang University School of Medicine in 1985 for PKU and CH. After extension of several diseases, PKU, Galactosemia, Maple syrup urine disease (MSUD), Homocystinuria (Hcy) and CH has been screened since 1991 for only those delivered from low income class mother at provincial public health care centers. Central and local governmental funded nationwide newborn screening program have been operated at the 70 small laboratories including two large non-profit medical foundation laboratories since 1997.

To evaluate the collective total incidence for disorders in the metabolism of amino acids, organic acids and fatty acids in Korea, metabolic disease detection laboratory in Seoul Medical Science Institute geared the incorporation of newborn screening using MS/MS to existing nationwide newborn screening program over last 2 years for more than twenty metabolic disorders (Table 1).

Pilot study is indispensable to satisfy most of the ethical, legal and social issues around patient, doctors, and metabolic specialist in General, local Hospital and related research laboratories. The informed consent allows the parents of newborns to "opt in" or to "decline" testing for the pilot screening.

We present our 2 years experience of metabolic screening by MS/MS including newborn screening, high risk screening and secondary confirmatory analysis since April 2001.

Experimental

1. Chemicals

Labeled acylcarnitines ($^{2}H_3$-carnitine, $^{2}H_3$-acetylcarnitine, $^{2}H_3$-propionylcarnitine, $^{2}H_3$-butrylcarnitine, $^{2}H_3$-isovalerylcarnitine, $^{2}H_3$-hexanoylcarnitine, $^{2}H_3$-octanoyl carnitine, $^{2}H_3$-decanoylcarnitine, $^{2}H_3$-dodecanoylcarnitine, $^{2}H_3$-tetradecanoylcarnitine, $^{2}H_3$-hexadecanoylcarnitine, $^{2}H_3$-octadecanoylcarnitine) and labeled amino acids (^{15}N,2-^{13}C-glycine, $^{2}H_4$-alanine, $^{2}H_8$-valine, $^{2}H_3$-leucine, $^{2}H_3$-methionine, $^{2}H_5$-phenylalanine, $^{13}C_6$-tyrosine, $^{2}H_3$-aspartate, $^{2}H_3$-glutamate, $^{2}H_2$-ornithine, $^{2}H_2$-citrulline, $^{2}H_4$,^{13}C-arginine) were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Other chemicals such as HPLC grade methanol, acetonitrile and acetic acid from Sigma-Aldrich Korea (Seoul, Korea) and Fisher Scientific Korea (Seoul, Korea).

Table 1. Metabolic Disorders Screened Using Tandem Mass Spectrometry

	Disorders
Organic aciduria	Propionic acidemia
	Methylmalonic acidemia
	Ethylmalonic encephalopathy
	Glutaric aciduria type I
	Isovaleric acidemia
	3-Methylcrotonylglycineuria
	Multiple carboxylase def.
	Methylmalonic aciduria with homocystinuria
Aminoacidopathy	Phenylketonuria
	Atypical phenylketonuria
	Homocysteinemia
	Hypermethioninemia
	Maple syrup urine disease
	Hypervalinemia
	Carbamylphosphate synthase def.
	Citrullinemia
	Argininosuccinic aciduria
	HHH syndrome
	Argininemia
	Tyrosinemia type 1
	Tyrosinemia type 2
	Tyrosinemia type 3
	Neonatal tyrosionemia
	Non-ketotic hyperglycinemia
	Hyperornithinemia
Fatty acid oxidation disorder	Carnitine uptake def.
	Carnitine palmitoyl transferase type 1 def.
	Acylcarnitine translocase def.
	Carnitine palmitoyl transferase type 2 def.
	Short chain acyl CoA dehydrogenase def.
	Short chain hydroxy acyl CoA dehydrogenase def.
	Medium chain acyl CoA dehydrogenase def.
	Multiple acyl CoA dehydrogenase def. (MADD)
	Riboflavin responsive MADD
	Long chain acyl CoA dehydrogenase def.
	Very long chain acyl CoA dehydrogenase def.
	Long chain hydroxy acyl CoA dehydrogenase def.
	Mitochondrial trifunctional protein def.
	B-Ketothiolase def.
	2,4-Dienoyl CoA reductase def.
	Hydroxy methyl glutaryl CoA lyase def.

2. ESI-MS/MS Conditions

MS/MS was performed on the API 2000 triple quadrupole mass spectrometry equipped with turbo electrospray ion source (PerkinElmer Life Science Inc. Boston, MA, USA). The two microliters of sample was delivered into the ESI source using micro-LC (Series 200, PerkinElmer Life Science Inc. Boston, MA, USA) and autosampler (Series 200, PerkinElmer Life Science Inc. Boston, MA, USA) without LC column. The mobile phase used was 80% acetonitrile. The flow rate was 40 μL/min with the total running time of 1.5 min.

The instrument settings were as follows : the turbo ion-spray interface was maintained at 200℃ with a nitrogen nebulization. The nitrogen was at a pressure of 15psi. The turbo ion-spray drying gas (N_2) was at a pressure of 40psi, the collision activated dissociation gas (CAD) was at a pressure of 6psi and curtain gas (CUR) was at a pressure of 25psi. The turbo ion-spray voltage was 5500V.

For the analysis of acylcarnitines (precursor 85 ion scan), parameter setting was as follows : declustering potentials (DP), 40V; focusing potential (FP), 391V; entrance potentials (EP), -9V; collision cell entrance potentials (CEP), 18V; collision energies (CE), 43V; collision cell exit potentials (CXP), 2V; deflector (DF), -150V and channel electron multiplier (CEM), 2000V. For the analysis of amino acids (neutral loss 102 scan), parameter setting as follows : DP, 25V; FP, 391V; EP, -8V; CEP, 12V; CE, 21V; and CXP, 5V. For the analysis of ornithine and citrulline (neutral loss 119 scan), parameter setting as follows : DP, 27V; FP, 391V; EP, -6V; CEP, 9V; CE, 29V; and CXP, 4V. For the analysis of glycine (neutral loss 56 scan), parameter setting as follows : DP, 23V; FP, 391V; EP, -7V; CEP, 6V; CE, 13V; and CXP, 10V. For the analysis of arginine (neutral loss 161 scan), parameter setting as follows : DP, 28V; FP, 391V;

EP, -9V; CEP, 14V; CE, 47V; and CXP, 9V. For the analysis of argininosuccinic acid (producit 459 ion scan), parameter setting as follows : DP, 81V; FP, 350V; EP, -9.5V; CEP, 18V; CE, 73V; and CXP, 8V.

3. Procedure

One spot of blood (3.2 mm diameter) was punched and delivered to 96 well-microplate (Costar, Cambridge, MA, USA). Extraction solvent, 150 μL of methanol containing isotopically labeled internal standards was added and shaken for 30 minutes at 30℃. The supernatant (75 μL) was transferred to a second plate and the wells were evaporated to dryness under nitrogen at 50℃. For butylation, 3 N butanolic hydrogen chloride (Regis Inc., IL, USA; 100 μL/each well) was added to the residue and heat at 65℃ for 15 min. The reactant was evaporated to dryness under nitrogen (40℃) and redissolve in acetonitrile/water (4 : 1). The solution was directly injected into turbo electrospray ion source of ESI-MS/MS.

Results and Discussion

The dried blood spots were generally taken 2-5 days after the infant's birth for newborn screening and were randomly taken from the children between 1 month and 20 years for high risk screening. The laboratory analysis of metabolic markers of disease began two day after the specimens were received over the country. The initial test results were available the next morning after the test; retesting of the specimens above the cut-off level was completed by 24 h. Thus, final results were generally available within 36 h after receipt of the specimen.

The annual birth of approximate 550,000 newborns was expected potential for the testing. Up until now approximately 31,000 newborns or infants and children at high risk were tested for organic, amino and fatty acid metabolism disorders. Each microtiter plate analyzed had 5 blanks and had 2 blood spot controls, and one of these controls contained markers with concentrations near the cut-off value. The precision of the mean of marker was as follows : acylcarnitines (C3, C8, C16), 11%; amino acids Leu, 10%, Orn, 18%, Met, 9%, Phe, 2%, Cit, 3% and Tyr, 16%. In this report, we describe the newborn, infants and children diagnosed as metabolic disorders over the past 2 years with either out-of-range (flagged) amino acid or acylcarnitine concentrations.

The metabolic disorders screened for organic acid, amino acid, and fatty acid metabolism, were listed in Table 1. The reference range of newborns (n=1,000) was presented in Table 2 and Table 3 for acylcarnitine and amino acid, respectively. The initial cut-off value was set as upper level of 99 percentile+2SD based on the range of 1,000 normal newborns and detected cases of metabolic disorders. The total number of newborn (24,700) and high risk group screened was 31,000 from April 2001 to March 2003. At the onset of

Table 2. Reference Value for Amino Acids with Blood Spot in Full-term Newborn

Amino acids	Newborn population (n=1,000) (Unit : μmol/L)	
	2SD	Reference range (1-99%)
Phenylalanine	12.5	22.9-95.6
Phe/Tyr	0.1	0.2-1.4
Methionine	6.3	15.2-53.6
Leu/Ileu	19.7	63.7-341.0
Valine	41.8	57.9-250.0
Citrulline	5.3	4.6-29.5
Cit/Phe	0.1	0.1-0.6
Cit/Tyr	0.1	0.0-0.4
Arginine	8.8	3.2-34.9
Tyrosine	15.7	30.8-218.0
Glycine	29.5	161.0-751.0
Ornithine	8.6	29.1-200.0
Alanine	46.7	110.0-452.0
Glutamate	30.0	146.0-704.0
Aspartate	22.1	17.6-172.0

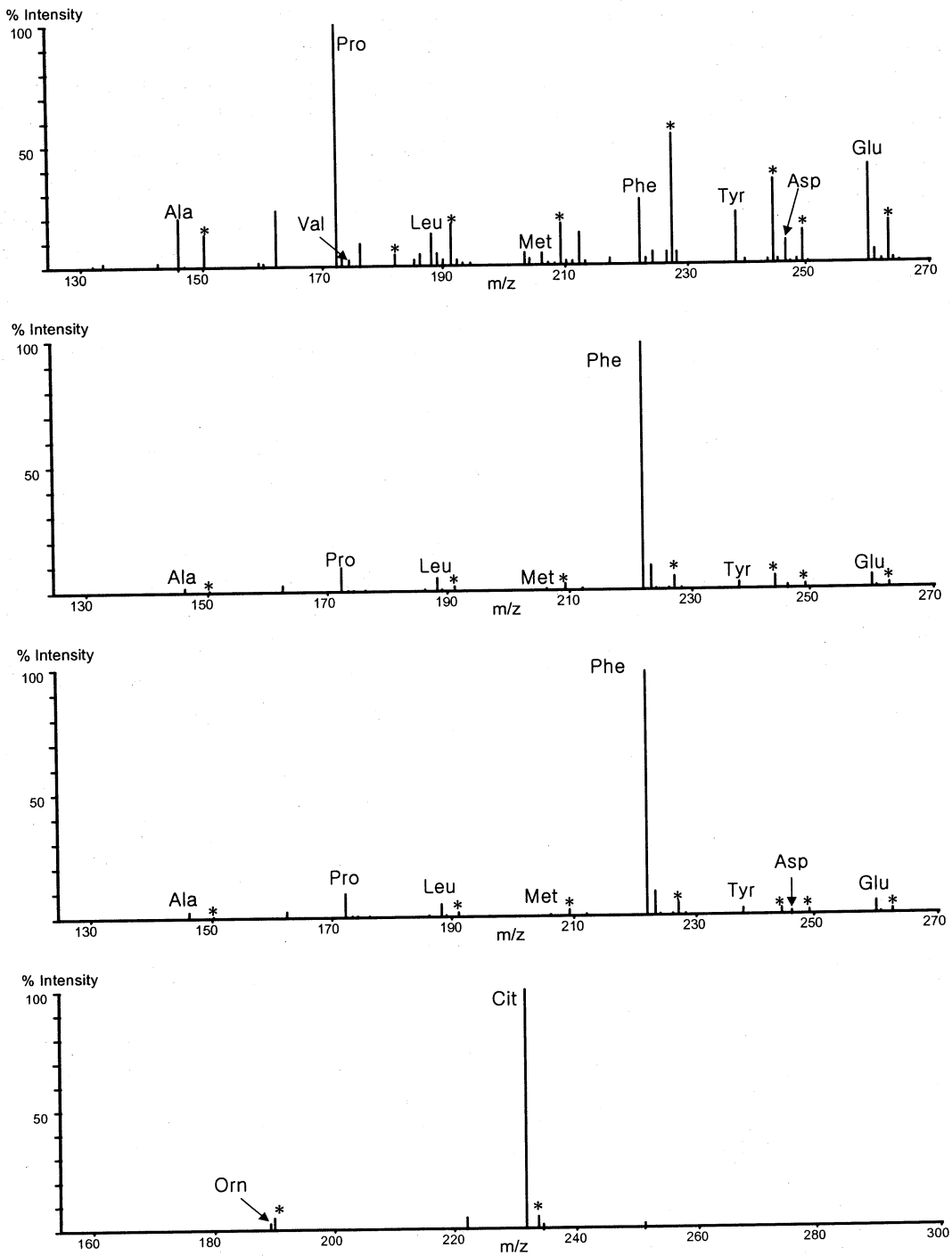

Fig. 1. Amino acid profile of (A) normal newborn, (B) PKU, (C) BH4 def-PKU and (D) citrullinemia.

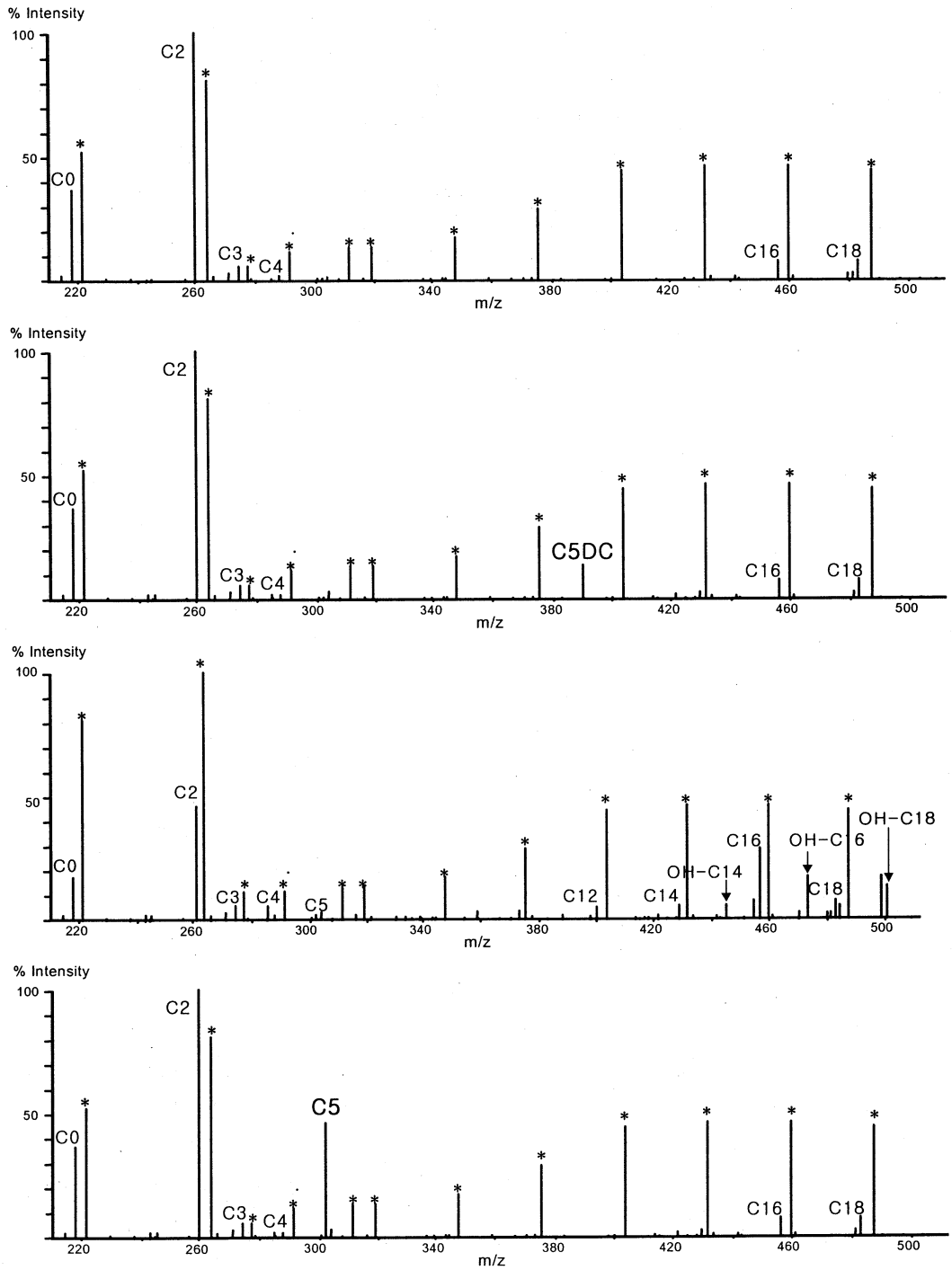

Fig. 2. Acylcarnitine profile of (A) normal newborn, (B) Glutaric aciduria type 1, (C) LCHAD deficiency and (D) Isovaleric aciduria.

Table 3. Reference Value for Acylcarnitines with Blood Spot in Full-term Newborn

Acylcarnitines	Newborn (n=1,000), Unit : μmol/L	
	2SD	Reference range (1-99%)
Free carnitine	20.55	4.760-59.30
Acetylcarnitine	4.34	2.150-22.70
Propionylcarnitine	1.22	0.306-7.820
C3/C2	0.45	0.036-1.507
Methylmalonylcarnitine	1.27	0.009-4.330
Butyrylcarnitine	0.19	0.058-0.701
C4/C2	0.05	0.005-0.144
C4/C3	0.11	0.019-0.641
Tiglylcarnitine	0.04	0.007-0.204
Isovalerylcarnitine	0.07	0.013-0.542
Glutarylcarnitine	0.04	0.007-0.120
C5/C2	0.02	0.002-0.114
C5/C3	0.04	0.005-0.595
3-OH-Isovalerylcarnitine	0.50	0.007-1.670
Adipylcarnitine	0.04	0.003-3.800
Hexanoylcarnitine	0.09	0.013-0.534
Octanoylcarnitine	0.08	0.012-0.228
Decadienoylcarnitine	0.03	0.005-0.145
Decenoylcarnitine	0.09	0.007-0.159
Decanoylcarnitine	0.13	0.009-0.281
Dodecanoylcarnitine	0.05	0.027-0.298
Tetradecadienoylcarnitine	0.03	0.005-0.140
Tetradecenoylcarnitine	0.04	0.004-0.157
C14 : 1/C16	0.05	0.002-0.148
3-OH-Tetradecanoylcarnitine	0.02	0.005-0.111
3-OH-Hexadecenoylcarnitine	0.02	0.005-0.119
3-OH-Hexadecanoylcarnitine	0.02	0.005-0.091
3-OH-Linoleylcarnitine	0.02	0.007-0.180
3-OH-Oleylcarnitine	0.01	0.004-0.075
3-OH-Stearoylcarnitine	0.01	0.003-0.089
Myristoylcarnitine	0.04	0.026-0.405
Hexadecenoylcarnitine	0.06	0.005-0.187
Palmitoylcarnitine	0.87	0.351-5.790
Linoleylcarnitine	0.23	0.005-0.620
Oleylcarnitine	0.50	0.019-1.860
Methyl-Glutarylcarnitine	0.065	0.004-5.570

the screening, the number of specimens was not high volume, however, at the end of last year it was dramatically increased. Diagnosed patient as metabolic disorders were as follows; Confirmed 28 (0.09%) multiple metabolic disorders (newborn/high risk) were as follows; 13 amino acid disorders [classical PKU (3/4), BH4 deficient-hyperphenylalaninemia (0/1), Citrullinemia (1/0), Homocystinuria (0/2), Hypermethioninemia (0/1), Tyrosinemia (1/0)], 8 organic acidurias [Propionic aciduria (2/1), Methylmalonic aciduria (0/1), Isovaleric aciduria (1/1), 3-methylcrotonylglycineuria (1/0), Glutaric aciduria typel (1/0)], 7 fatty acid oxidation disorders [LCHAD def. (2/2), Mitochondrial TFP def. (0/1), VLCAD def. (1/0), LC3KT def. (0/1)](Fig. 1 & 2).

Timing of these follow-up tests can be critical for many disorders because the abnormal markers may not be informative if the infant is not metabolically decompensated. In addition, a decrease the acylcarnitines is expected when regular feeding are established.

Newborn Screening by MS/MS is well suited for high-throughput mass screening where a shorter analytical time is highly desirable and early diagnosis is indispensable for the life quality of newborn if affected. Despite our limited experience, MS/MS newborn screening will give an insight to determine the implementation of MS/MS and future strategy of newborn screening.

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