

Review article

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Screening newborns for metabolic disorders based on targeted metabolomics using tandem mass spectrometry

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The main purpose of newborn screening is to diagnose genetic, metabolic, and other inherited disorders, at their earliest to start treatment before the clinical manifestations become evident. Understanding and tracing the biochemical data obtained from tandem mass spectrometry is vital for early diagnosis of metabolic diseases associated with such disorders. Accordingly, it is important to focus on the entire diagnostic process, including differential and confirmatory diagnostic options, and the major factors that influence the results of biochemical analysis. Compared to regular biochemical testing, this is a complex process carried out by a medical physician specialist. It is comprised of an integrated program requiring multidisciplinary approach such as, pediatric specialist, expert scientist, clinical laboratory technician, and nutritionist. Tandem mass spectrometry is a powerful tool to improve screening of newborns for diverse metabolic diseases. It is likely to be used to analyze other treatable disorders or significantly improve existing newborn tests to allow broad scale and precise testing. This new era of various screening programs, new treatments, and the availability of detection technology will prove to be beneficial for the future generations.

Keywords: Tandem mass spectrometry, Inherited metabolic disease, Early diagnosis, Differential diagnosis, Newborn screening

Introduction

Inborn errors of metabolism are a heterogeneous group of metabolic disorders caused by an enzyme defect in a metabolic pathway, leading to dysfunction of metabolism and the accumulation of toxic intermediate metabolites. Newborn screening test (NST), introduced in the 1960s by scientist Robert Guthrie, is used to screen newborn and infants for metabolic disorders using classical methods that includes Guthrie assay, bacterial inhibition assay, and enzyme immune assay. Later, tandem mass spectrometry had been introduced as combined with fast atom bombardment and electrospray ionization¹⁻⁷⁾, recently with high resolution chromatography⁸⁾ and direct analysis in real time⁹⁾ mass spectrometry for NST¹⁻¹⁰⁾. For the recent two decades, tandem mass spectrometry has been applied to screen over 60 different defined metabolic disorders, thus enabling their early diagnosis and treatment^{2,11,12)}. The specificity and sensitivity of tandem mass spectrometry method could be up to 99.995% and 99%, respectively, for most organic acidemias, amino acid disorders, and fatty acid oxidation disorders as well as extended program comprising of Fabry, Pompe, and mucopolysaccharidosis-I¹³⁻¹⁵⁾. Though the technical advances achieved in mass spectrometry have facilitated the use of dozens of various biochemical markers, special care needs to be taken in interpreting output data of the tested marker metabolites because false interpreting

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can lead to a misdiagnosis^{12,16-18)} to metabolic disorders more thoroughly, and speed up the process of early diagnosis and treatment^{12,16-18)}.

Metabolites as a targeted marker on dried blood-spot and inborn metabolic diseases

One abnormal finding in tandem mass spectrometry points to several different metabolic diseases as shown in Table 1¹⁹⁻²¹⁾. One of the reasons for such abnormal result is that the acylcarnitine isomers cannot be differentiated by tandem mass spectrometry. Medication can also trigger changes in the body that mimic a metabolic disease. For these reasons, tandem mass spectrometry is often performed for initial mass screening, and second alternate tests, such as urine organic acid analysis and plasma amino acid analysis, are additionally required for the confirmatory follow-up diagnostic test¹⁹⁻²¹⁾.

Acylcarnitine as a marker for fatty acid oxidation disorder and organic aciduria

It is necessary to differentiate between systemic carnitine deficiency (carnitine uptake deficiency, CUD) and secondary carnitine deficiency in case of low free carnitine (C0)^{18,20)}. CUD is characterized by increased renal excretion and low concentrations of C0 and all acylcarnitines. CUD is a congenital deficiency of carnitine/organic cation transporter 2 characterized by defects in the beta-oxidation pathway due to low carnitine levels. Though the diagnostic marker for CUD is decreased C0 value, the possibility of false negative cannot be ruled out, especially in long-stored samples due to increase in C0 values over time. However, there is a report that systemic carnitine deficiency is hardly influenced by hydrolysis because of the low level of the total acylcarnitine^{19,22)}. On the other hand, there are cases where secondary carnitine deficiency is accompanied by increase of a specific acylcarnitine (increase of 3-hydroxyisovaleryl carnitine [C5-OH], C5, and

Table 1. Metabolites and suspicious metabolic disease

Metabolites	2nd Testing recommended	Suspicious metabolic disease
Phe		PKU, citrin deficiency (citrulline ↑)
[Leu+Ile] & Val	AA (plasma), OA (urine)	MSUD, ketosis
Met	AA (plasma), ammonia (blood)	Homocystinuria, MAT deficiency, citrin deficiency (citrulline (high))
Cit	AA (plasma), ammonia (blood)	Citrullinemia I, argininosuccinic aciduria, citrin deficiency
Cit (low)	ammonia (blood)	OTC deficiency, CPS1 deficiency
Arg	AA (plasma)	Hyperargininemia, citrin deficiency(citrulline (high))
Tyr	OA(urine)	Hypertyrosinemia (if succinylacetone (↑) → type 1), liver dysfunction
C0 (low)	Removal rate of carnitine	systemic carnitine deficiency, secondary carnitine deficiency
C0	OA (urine)	CPT1 deficiency, tissue degradation (hypoxia)
C2	OA (urine)	Ketosis
C2 (low)		(general fatty acid oxidation problem)
C3 ³⁸⁾	OA (urine)	Methylmalonic aciduria, propionic aciduria
C4	OA (urine)	IBD deficiency, SCAD deficiency, glutaric aciduria type 2
C4-OH	OA (urine)	SCHAD (HAD) deficiency, ketosis
C5	OA (urine)	Isovaleric aciduria, under administration of pivaloyl containing antibiotics, 2MBD deficiency
C5-DC	OA (urine)	Glutaric aciduria type 1, glutaric aciduria type 2
C5-OH	OA (urine)	Complex carboxylase deficiency, methylcrotonylglycineuria, HMG uria, beta-ketothiolase deficiency, MCG-CoA hydrolase enzyme deficiency, biotin deficiency
C8	OA (urine)	MCAD deficiency (C8>C10), glutaric aciduria type 2 (C8<C10)
C10–C12	OA (urine)	Glutaric aciduria type 2
C14:1	OA (urine)	VLCAD deficiency, ketosis
C16	OA (urine)	CPT2 deficiency, CACT deficiency, VLCAD deficiency, glutaric aciduria type 2
C16-OH	OA (urine)	TFP/LCHAD deficiency
C18:1(C18)	OA (urine)	CPT2 deficiency, CACT deficiency, VLCAD deficiency, glutaric aciduria type 2
C18:1-OH	OA (urine)	TFP/LCHAD deficiency
C16, C18 (low)	OA (urine)	CPT1 deficiency

PKU, phenylketonuria; AA, amino acid analysis; OA, organic acid analysis; MSUD, maple syrup urine disease; MAT, methionine adenosyl transferase; OTC, ornithine transcarbamylase; CPS1, carbamoyl phosphate synthetase I; IBD, isobutyryl CoA dehydrogenase; SCAD, short chain acyl-CoA dehydrogenase; SCHAD, short chain 3-hydroxyacyl-CoA dehydrogenase; 2MBD, 2-methylbutyryl-CoA dehydrogenase; HMG, 3-hydroxy-3-methyl-glutaryl-CoA reductase; MCG, methylcrotonylglycine; MCAD, medium-chain acyl-coenzyme A dehydrogenase; VLCAD, very long-chain acyl CoA dehydrogenase; CPT, carnitine palmitoyltransferase; CACT, carnitine acylcarnitine translocase; TFP, trifunctional protein; LCHAD, long-chain 3-hydroxyacyl-CoA dehydrogenase.

C3 in methylcrotonylglycinuria, isovaleric aciduria, and methylmalonic aciduria, respectively). While screening newborns if the mother has systemic carnitine deficiency, the newborn exhibits low C0 concentration even when unaffected by the metabolic disease. Moreover, it has been reported that if the mother is affected by glutaric aciduria type 1, secondary carnitine deficiency can occur and the newborn can also show low C0 concentration. In many organic aciduria patients, secondary free carnitine deficiency occurs with elevation of specific acylcarnitine. For this reason, long-stored samples are likely to fail to confirm secondary carnitine deficiency due to the ease of hydrolytic degradation²².

If muscle tissue proteolysis occurs in carnitine palmitoyltransferase I (CPT1) deficiency and hypoxic shock, large quantity of C0 contained in the tissue is released into the blood, thus, increasing the blood free carnitine (C0) concentration. This is very often concurrent with the elevation of C3 and C4. The characteristic of CPT1 deficiency are increased C0 concentration, decreased long-chain acylcarnitine, and increased C0/[C16+C18] ratio. However, samples unaffected with CPT1 deficiency can present a pattern similar to that of CPT1 deficiency due to hydrolysis of acylcarnitine^{22,23}.

In case of concurrent increase in 3-hydroxybutyryl carnitine (C4-OH) and C2, the odds are high that 3-hydroxylactate also increases, and ketosis can be suspected. Since C2 levels increase in short chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD)/3-hydroxyacyl-CoA dehydrogenase (HAD) deficiency, increased C4-OH/C2 ratio is used as a marker for screen SCHAD deficiency. Additionally, by the time SCHAD deficiency is manifested, C6-OH increases concurrently. However, care should be taken when a nonderivatization method is used in tandem mass spectrometric analysis it is expressed by the increase of glutarylcarnitine (C5-DC) instead of elevation of C6-OH^{22,24}.

An elevation of C5 is indicative of the presence of one of these four isomers; isovalerylcarnitine, 2-methylbutyrylcarnitine, pivaloylcarnitine, and valerylcarnitine. Isovalerylcarnitine increases in isovaleryl aciduria, and so does 2-methylbutyryl-CoA dehydrogenase deficiency 2-methylbutyrylcarnitine^{16,25-27}. A long-term administration of oral antibiotics and some parenteral antimicrobials, it leads to increase in pivaloylcarnitine. They can be differentiated by urine organic acid analysis, whereby slight increase of C5 can occur for unclear reasons^{23,25}.

High C5-DC level requires a differential diagnosis (glutaric acidemia types I and II) by urine organic acid analysis. Newborn patient with glutaric aciduria type 1 excretes abnormal quantities of glutaric acid and 3-hydroxyglutaric acid whereas glutaric aciduria type 2, 2-hydroxyglutaric acid. However, elevation of glutaric acid excretion in infants and young children is not always clearly noticeable in glutaric aciduria type 1. When ketone increases, C5-DC levels may be slightly elevated²³.

If C5-OH increases, diagnosis is simple because urine exhibits a distinctively abnormal metabolite excretion in urine organic acid analysis. If a preterm infant is fed special formula devoid of

biotin, C5-OH increases due to biotin deficiency. While biotin injection can rapidly lower the serum C5-OH level, C5-OH accumulated in the red blood cells cannot transfuse out and C5-OH level continues to rise depending on red blood cell survival time. The urine organic acid analysis of a preterm infant with biotin deficiency reveals the elevated level of 3-hydroxyisovaleric acid. If the mother has methylcrotonylglycinuria, the maternal C5-OH is transfused to the fetus and gets accumulated in the red blood cells. In such a case, C5-OH accumulated in the red blood cells is detected in the dried blood-spot analysis, resulting in a false positive result²⁵. For confirmatory diagnosis, maternal urine organic acid analysis is necessary²³.

C5-OH increases at an extremely low rate in mild beta-ketothiolase deficiency unless symptoms are acute.

In most of such cases, mothers are not aware of their pathological conditions. They usually exhibit mild symptoms or remain asymptomatic depending on environmental factors during their childhood and youth. Although found through newborn screening the maternal blood free carnitine level is very low, symptoms may or may not manifest depending on any given occasion. Hypocarnitinemia may be prevented by carnitine intake as needed²³.

Increase in C6, C8, C10, with C8 most markedly, indicates medium-chain acyl-coenzyme A dehydrogenase (MCAD) deficiency²⁸⁻³⁰. Slightly elevated C8 of a neonate is also presented in MCAD deficiency carrier. If C10 is higher than C8, glutaric aciduria type 2 is suspected. Elevated levels of C8 and C10 also appear in preterm infants fed with preterm formula in the form of medium-chain triglyceride (MCT) oil²⁵, which can be confirmed with enzyme activity assay and gene analysis²².

Specific diagnostic marker, C14:1 carnitine increases in very long-chain acyl CoA dehydrogenase (VLCAD) deficiency. Extremely severe case of VLCAD deficiency is associated with more marked increase in C16 and C18:1 than in C14:1 in both serum and dried blood-spot analysis. Carnitine palmitoyl transferase 2 (CPT2) deficiency and carnitine acylcarnitine translocase (CACT) deficiency involves elevated C16 and C18:1³¹, but no change in C14:1 levels, and can thus be easily differentiated from VLCAD deficiency. C14:1 is slightly elevated in ketosis as well, which involves increase in C2, thus lowering the C14:1/C2 ratio. For confirmatory diagnosis, enzyme activity assay and gene analysis should be performed²².

An elevation of C16 and C18:1 (or C18) point towards CPT 2 deficiency and CACT deficiency. Since certain levels of C16 and C18:1 are found in blood-spot even in healthy conditions, reflecting the red blood cell concentration, only a remarkable elevation can be considered to be abnormal³¹. Increase in C14:1 along with considerably elevated C16 and C18:1 levels is indicative of VLCAD deficiency, and not CPT 2 or CACT deficiency. If C2 level is increased along with slightly elevated C16 and C18:1 levels, odds for ketosis are very much high²².

Low level of C16 and C18:1 (C18) in dried blood-spot analysis is indicative of CPT1 deficiency. Decreased C16 and C18:1 (C18) levels can also be found in neonates who have recovered from extremely ill conditions such as suspended animation, but

the phenomenon is transient and the levels are often normalized in re-examination²²⁾.

An elevation of C16-OH and C18:1-OH is a condition found in mitochondrial trifunctional protein (TFP)/long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency. In LCHAD deficiency C18-OH level increases as well as, at a higher rate, C18:1-OH increases. Patients with severe type of TFP/LCHAD deficiency exhibit significantly elevated C16-OH and C18:1-OH levels, but the degree of increase fluctuates, and severity cannot be predicted only by the degree of increase of C18:1-OH levels. If C2 is increased along with C16-OH and C18:1-OH, it is most likely caused due to ketosis^{6,18,22,23,32)}.

Amino acids as a marker for amino acid metabolism disorders

An elevated level of tyrosine detected in newborn screening by tandem mass spectrometry is usually due to transient neonatal hypertyrosinemia. To differentiate, tyrosinemia type 1 can be diagnosed by presence of succinylacetone in serum or urine³⁴⁾. Although the patient has tyrosinemia type 1, tyrosine concentration does not always high levels as in neonate³²⁾.

An elevated phenylalanine (Phe) is indicative of phenylketonuria³³⁾. Additionally, newborns and infants with neonatal intrahepatic cholestasis caused by citrine deficiency may have elevated Phe levels concomitant with increase in citrulline. Phe may increase along with tyrosine and methionine levels, secondary to liver dysfunction³⁾.

An elevated citrulline appears in citrullinemia type 1, argininosuccinic aciduria, and citrine deficiency. Argininosuccinic acid increases concurrently during argininosuccinic aciduria in urine. Patients with neonatal intrahepatic cholestasis caused by citrine deficiency have an elevated amino acid level, besides citrulline, accompanied by occasional increase in serum galactose³²⁾.

Low serum citrulline concentration is associated with ornithine transcarbamylase (OTC) deficiency³⁴⁾ and carbamoyl phosphate synthetase I (CPS1) deficiency concomitant with hyperammonemia. However, in newborn screening by tandem mass spectrometry, these diseases have been reported even in cases with normal citrulline concentration³²⁾.

An elevated leucine, isoleucine, and valine appear in maple syrup urine disease (MSUD)³⁵⁾. If it is concomitant with C2 increase, odds are high that it is due to the ketosis-related expedited catabolism. For differential diagnosis, serum alloisoleucine analysis and urine organic acid analysis can be used, the presence of alloisoleucine is essential in MSUD. Enzyme activities could be measured for confirmatory diagnosis³²⁾.

Conditions influencing numeric value of metabolites

In quantifying targeted metabolites using tandem mass spectrometry, many factors influence the results of acylcarnitine

and amino acids^{7,18,31)}. In newborn screening, level of metabolites can be moderately changed in accordance with changed biological metabolic process by medical treatment or influenced by specimen status based on various external factors^{32,36)}.

As for sample storage environment if samples are stored in high temperature with humidified conditions, levels of measured metabolites are likely to yield inaccurate values. Especially under such environments level of free carnitine tend to increase and acylcarnitine decrease. Altered metabolite value can be minimized by storing the dried blood samples in a freezer (or refrigerator), preferably in an airtight bag. If possible fresh samples should be used except for unavoidable cases such as postmortem examination after a sudden death^{37,38)}.

Regarding effect of pH acylcarnitine becomes more unstable in a high pH than in low pH environment. In urine samples stored prolonged period, the pH tends to exceed pH >8.0, the influence of pH should be accounted for. Excessive hemolysis increase in long-chain acylcarnitine level due to excessive hemolysis³⁷⁾. Hemolysis induced by "squeezing blood collection" does not pose problems, but samples with excessive hemolysis can yield findings similar to long-chain fatty acid metabolism disorder³⁸⁾.

Short-chain acylcarnitine (C3–C4) is more prone to hydrolysis than long- (C12–C18) and medium-chain (C5–C10) acylcarnitine species²⁵⁾. Therefore, propionic acidemia and methylmalonic acidemias, for which C3 acts as biomarker, may not be detected in dried blood spot stored at room temperature for over one year. In contrast, long-chain fatty acid metabolism disorder was diagnosable without difficulties in blood-spot samples stored for over three years^{32,37,38)}.

The measured values of C6, C8, C10, and C12 increase while administering MCT oil. The ratio of C8/C10 can be used to differentiate such increases due to MCAD deficiency. Many reported cases proved that urine organic acid analysis is efficient for differentiation between MCT oil and MCAD deficiency^{32,37)}.

Some total parenteral nutrition (TPN) products are rich in branched chain amino acids, leading to test values similar to that in MSUD²⁰⁾. For differential diagnosis, amino acid ratios, such as Phe/Tyr, Met/Phe, and Leu/Ala ratios, are important markers³²⁾. In some cases, detailed secondary confirmatory testing is required. Most importantly, specimen should be collected before beginning parenteral nutrition as much as possible. Moreover, TPN contains substances similar to C8 and C12, whose levels may look elevated²⁾. Since TPN does not contain carnitine, babies under a long-term parenteral nutrition therapy can develop carnitine deficiency²⁵⁾.

Some antibiotics such as ampicillin and cefotaxime can induce increase in the level of acylcarnitines (C5, C14:1, and C16:1-OH). The level of C5 can increase by consuming antibiotics containing the pivaloyl functional group, such as cefcapenepivoxil, cefditoren pivoxil, and ceftam pivoxil²⁴⁾. There have been cases in which C5 levels increased after the administration of ciberestat. These components can be differentiated by urine organic acid analysis^{32,38)}.

If the mother has a carrier for inborn metabolic disorder,

blood-spot result of her healthy neonate can exhibit abnormal findings in newborn screening. Reported metabolic diseases like these cases are associated with asymptomatic systemic carnitine deficiency, methylcrotonylglycinuria, 3-methylglutaconic aciduria, glutaric aciduria type 1, and vitamin B12 deficiency. Neonates from vegetarian mothers show decreased free carnitine levels due to the mother's status of low carnitine level resulted from vegetarianism^{32,39}.

In addition, other conditions influencing numeric value of metabolites are in a poorly fed neonate and cholestasis neonate³⁸⁻⁴⁰.

Newborns with low birth weight often exhibit transient neonatal tyrosinemia. In preterm and low-birth weight newborns, implications of measured values are often unclear and no defined reference values have been established so far³⁹. When interpreting the test values for preterm and low-birth weight newborns, it should also be borne in mind that nonspecific pathological manifestations can also affect measured value^{15,18,39}.

The blood test is generally performed when a baby is 24 to 48 hours old after enough feeding. This timing is very important because certain conditions may go undetected if the blood sample is drawn before 24 hours of age. Newborn screening does not confirm a baby has a certain condition. If a positive screen is detected, parents will be notified immediately and follow-up testing will be done.

Conclusions

Three decades have passed since the first newborn screening has started in South Korea in 1985. To fulfill the goal of newborn screening, early diagnosis and treatment of metabolic diseases, integrated interdisciplinary collaboration between specialist pediatricians and expert scientist of related fields is essential, especially for accurate measurements and interpretations of biochemical data obtained by tandem mass spectrometry. Thereby reflecting each patient's meticulously examined physical status, enables customized differential diagnosis of inherited metabolic disorders.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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