Newborn Screening by Tandem Mass Spectrometry: Gaining Experience

Major expansion of newborn screening for inherited metabolic disorders is taking place across the US and around the world as newer analytical technology is applied. Historically, each disorder to be screened required a separate test with associated costs and requirement for a portion of the dried-blood-spot specimen from a heel stick. This limitation of the existing tests was partially responsible for the limitation of mandated newborn screening in the US to a small number of disorders (usually three to seven, depending on the state).

The technique of tandem mass spectrometric (MS) analysis of dried-blood spots was first proposed for newborn screening in 1990 by Millington et al. *(1)*. Using ionization techniques of fast atom bombardment or liquid secondary ionization with tandem MS, they simultaneously determined a large number of acylcarnitines as an acylcarnitine profile. This allowed newborn screening for numerous inherited fatty acid oxidation and organic acid disorders by a single procedure. Tandem MS was extended to amino acids, including phenylalanine, the screening target for detection of the phenylketonuria (PKU) test *(2–4)*, and to other disorders, with several such tests described in these pages during the last 8 years *(3, 5–9)*. The development and application of electrospray ionization (ESI) tandem MS with its ability to be automated made high-volume tandem MS screening for amino acid, organic acid, and fatty acid metabolic disorders practical by the mid-1990s *(10–12)*.

Automated ESI tandem MS newborn screening of amino acids and acylcarnitines extracted from a single punch of a dried-blood spot, with stable-isotope-labeled internal standards and derivatization to butyl esters, can be performed with 2–4 min of instrument time. This single test is capable of detecting ~20–40 inherited metabolic disorders, depending on which analytes are measured and how different disorders are defined *(10–13)*. The number of disorders is based on experience with detection of increased metabolites in blood from children affected with these disorders, although not all have been demonstrated to be detectable in the neonate. The large increase in the number of inherited metabolic disorders detectable in the newborn period because of tandem MS screening greatly extends the possibilities of early, generally presymptomatic, diagnosis and treatment to minimize morbidity and mortality for many affected children. The aggregate incidence of disorders detectable by tandem MS (including PKU and other amino acid disorders that are currently screened for in some states) was reportedly ~1 in 3400 newborns for 168 000 newborns screened in Pennsylvania and North Carolina [summarized in *(14)*], 1 in 4700 for 137 000 babies screened in Australia *(13)*, and 1 in 3800 in >160 000 babies screened in New England by Zytkovicz et al. *(15)* as described in this issue of *Clinical Chemistry*.

These initial publications of the experiences of large-scale newborn screening for metabolic disorders by tandem MS also emphasize some of the complications of this expansion: (*a*) establishment of appropriate cutoffs for the large number of analytes to minimize false negatives without an excessive number of false positives; and (*b*) determination of a definitive diagnosis for an infant with an abnormal increase detected by tandem MS screening.

False positives cause parental anxiety and are expensive in terms of professional time and effort to obtain repeat specimens for retesting and follow-up *(16)*. The first publication of a computer algorithm for tandem MS newborn screening set the upper limit of the reference interval at the 99.5 percentile, which would give 0.5% positives for each analyte, which is reasonable for a single analyte test and disorder *(12)*. When screening for 20–40 disorders (analytes) in a single test (assuming that each is independent of the others), the aggregate incidence of false positives would be as high as 18%, whereas the true positives would be ~0.03%. To minimize false positives, Zytkovicz et al. *(15)* generally set the abnormal flags at the 99.98 percentile, so that only 0.02% would be flagged positive for each analyte, making the false-positive rate (for ~20 independent tests) ~0.4% with a true-positive rate of 0.03%. This minimizes false positives but raises some concern about possible false negatives because these flagging values were 5–13 SD above the normal mean for the analytes.

Zytkovicz et al. *(15)* point out that different cutpoints need to be established for different subpopulations of newborns. Only 5% of their newborns had very low birth weights or were in neonatal intensive care units, but they accounted for 50% of the false positives. Optimal discrimination may require determination of cutpoints for a larger number of subpopulations. These might include full-term, premature or low birth weight, very low birth weight, very low birth weight on total parenteral nutrition, specimen collection at <24 h of age (early discharge), and collection at 1–3 days of age. Additionally, blood concentrations of some analytes change with age, requiring different decision limits for repeat specimens obtained many days or weeks after birth to investigate a positive initial result in the neonate.

Another challenge illustrated in the report by Zytkovicz et al. *(15)* is confirmation of a diagnosis suggested by an abnormal screening result. Tandem MS is sometimes considered to provide definitive diagnoses, as is true for some disorders. In medium-chain acyl-CoA dehydrogenase deficiency (MCAD), the pattern of increased acylcarnitines is diagnostic. Even in this case, molecular analyses of mutations in the MCAD gene suggest that different mutations can produce different degrees of metabolite increases in affected babies *(17)*. Many of the abnormal analyte increases found in tandem MS screening are not pathognomonic of a single disorder and can be produced

by several different genetic disorders. In these cases, additional, more-specific diagnostic tests are required.

Some of the lack of specificity of the tandem MS results reflects the inability of the technique to distinguish isobaric compounds. For example, isobutyrylcarnitine and butyrylcarnitine have the same mass and are detected as one analyte (C4-acylcarnitine). Increased C4-acylcarnitine may reflect either of two very different disorders: isobutyryl-CoA dehydrogenase deficiency in the catabolic pathway of valine (increased isobutyrylcarnitine) or short-chain acyl-CoA dehydrogenase deficiency in fatty acid β-oxidation (increased butyrylcarnitine). Differential diagnosis requires additional tests, such as urinary organic acids, DNA mutation analysis, determination of the integrity of different metabolic pathways in intact cells, or assay of individual enzyme activities in cells. In other cases, different inherited disorders produce increases in the same abnormal compound. An example is increased propionylcarnitine (C3-acylcarnitine), a finding that requires differentiation of several disorders: propionyl-CoA carboxylase deficiency, methylmalonyl-CoA mutase deficiency, several cobalamin disorders, and even dietary deficiency of vitamin B_{12}. This requires the expertise of clinical and biochemical geneticists and a variety of diagnostic tests.

The experience presented by Zytkovicz et al. *(15)* in this issue of *Clinical Chemistry*, as well as the experience of others *(12, 13)*, is valuable for pointing out some of the complications that need to be addressed as tandem MS newborn screening is implemented in more and more states for an increasing number of metabolic disorders. Resolving the issues of establishing appropriate reference intervals for subpopulations of newborns, determining limits to minimize false positives and false negatives, developing protocols for the differential diagnosis of suspected disorders, compilation of incidences of disorders, and documentation of outcomes for validation of the programs will require the sharing of information among screening laboratories and their genetic consultants. This goal is being encouraged and greatly assisted by the collaborative efforts of the Genetic Services Branch of the Maternal and Child Health Bureau in the Health Resources and Services Administration (HRSA) in Washington, the CDC in Atlanta, and the National Newborn Screening and Genetic Resource Center (NNSGRC) in Austin *(18)*.

References

1. Millington DS, Kodo N, Norwood DL, Roe CR. Tandem mass spectrometry: a new method for acylcarnitine profiling with potential for neonatal screening for inborn errors of metabolism. J Inherit Metab Dis 1990;13:321–4.
2. Millington DS, Kodo N, Terada N, Roe D, Chase DH. The analysis of diagnostic markers of genetic disorders to human blood and urine using tandem mass spectrometry with liquid secondary ion mass spectrometry. Int J Mass Spectrom Ion Proc 1991;111:211–28.
3. Chace DH, Millington DS, Terada N, Kahler SG, Roe CR, Hofman LF. Rapid diagnosis of phenylketonuria by quantitative analysis for phenylalanine and tyrosine in neonatal blood spots by tandem mass spectrometry. Clin Chem 1993;39:66–71.
4. Chace DH, Sherwin JH, Hillman SL, Lorey F, Cunningham GC. Use of phenylalanine-to-tyrosine ratio determined by tandem mass spectrometry to improve newborn screening for phenylketonuria of early discharge specimens collected in the first 24 h. Clin Chem 1998;44:2405–9.
5. Chace DH, Hillman SL, Millington DS, Kahler SG, Roe CR, Naylor EW. Rapid diagnosis of maple syrup urine disease in blood spots from newborns by tandem mass spectrometry. Clin Chem 1995;41:62–8.
6. Chace DH, Hillman SL, Millington DS, Kahler SG, Adam BW, Levy HL. Rapid diagnosis of homocystinuria and other hypermethioninemias from newborns' blood spots by tandem mass spectrometry. Clin Chem 1996;42:349–55.
7. Chace DH, Hillman SL, Van Hove JLK, Naylor EW. Rapid diagnosis of MCAD deficiency: quantitative analysis of octanoylcarnitine and other acylcarnitines in newborn blood spots by tandem mass spectrometry. Clin Chem 1997;43:2106–13.
8. Jones PM, Quinn R, Fennessey PV, Tjoa S, Goodman SI, Fiore S, et al. Improved stable isotope dilution-gas chromatography-mass spectrometry method for serum or plasma free 3-hydroxy-fatty acids and its utility for the study of disorders of mitochondrial fatty acid β-oxidation. Clin Chem 2000;46:149–55.
9. Ito T, van Kuilenburg ABP, Bootsma AH, Haasnoot AJ, van Cruchten A, Wada Y, van Gennip AH. Rapid screening of high-risk patients for disorders of purine and pyrimidine metabolism using HPLC-electrospray tandem mass spectrometry of liquid urine or urine-soaked filter paper strips. Clin Chem 2000;46:445–52.
10. Rashed MS, Ozand PT, Bucknall MP, Little D. Diagnosis of inborn errors of metabolism from blood spots by acylcarnitines and amino acids profiling using automated electrospray tandem mass spectrometry. Pediatr Res 1995;38:324–31.
11. Johnson AW, Mills K, Clayton PT. The use of automated electrospray ionization tandem MS for the diagnosis of inborn errors of metabolism from dried blood spots. Biochem Soc Trans 1996;24:932–8.
12. Rashed MS, Bucknall MP, Little D, Awad A, Jacob M, Alamoudi M, et al. Screening blood spots for inborn errors of metabolism by electrospray tandem mass spectrometry with a microplate batch process and a computer algorithm for automated flagging of abnormal profiles. Clin Chem 1997;43:1129–41.
13. Wiley V, Carpenter K, Wilcken B. Newborn screening with tandem mass spectrometry 12 months' experience in NSW Australia. Acta Paediatr Suppl 1999;88:48–51.
14. Sweetman L. Newborn screening by tandem mass spectrometry (MS-MS). Clin Chem 1996;42:345–6.
15. Zytkovicz TH, Fitzgerald EF, Marsden D, Larson CA, Shih VE, Johnson DM, et al. Tandem mass spectrometric analysis for amino, organic, and fatty acid disorders in newborn dried blood spots: a two-year summary from the New England Newborn Screening Program. Clin Chem 2001;47:1945–55.
16. Levy HL. Newborn screening by tandem mass spectrometry: a new era. Clin Chem 1998;44:2401–2.
17. Andresen BS, Dobrowolski SF, O'Reilly L, Muenzer J, McCandless SE, Frazier DM, et al. Medium-chain acyl-CoA dehydrogenase (MCAD) mutations identified by MS/MS based prospective screening of newborns differ from those observed in patients with clinical symptoms: identification and characterization of a new, prevalent mutation that results in mild MCAD deficiency. Am J Hum Genet 2001;68:1408–18.
18. National Newborn Screening and Genetics Resource Center. http://genes-r-us.uthscsa.edu (Accessed September 22, 2001).

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