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Automated Tandem Mass Spectrometry for Mass Newborn Screening for Disorder in Fatty Acid, Organic Acid, and Amino Acid Metabolism

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The purpose of newborn screening is to accurately identify those individuals with inherited metabolic disorders at an age when early medical intervention can prevent or significantly reduce associated clinical symptoms such as mental retardation, acute metabolic crisis, seizures, or even early infant death. Newborn screening for inborn errors of metabolism using filter-paper blood specimens had its origin in the early 1960s with the development of the Guthrie bacterial inhibition assay for the detection of elevated phenylalanine associated with phenylkentonuria. It is estimated that more than 200 million newborn have been screened throughout the world using this simple bacterial inhibition assay for phenylketonuria and the filter-paper blood specimen. As new tests for additional metabolic conditions have been developed over the years, they too have been performed on the same filter-paper blood specimen. The different screening methodologies include radioimmunoassays, enzyme immunoassays, isoelectric focusing, high-performance liquid chromatography, fluorometry, and direct enzyme assays.

In general, each of these screening tests is directed to the diagnosis of a single metabolic disorder. This has resulted in the addition of routine screening for galactosemia, sickle cell hemoglobinopathies, maple syrup urine disease, homocystinuria, congenital adrenal hyperplasia, biotinidase deficiency, and cystic fibrosis by many laboratories in the United States and throughout the world. The typical screening laboratory today screens for phenylketonuria and congenital hypothyroidism plus two to four additional disorders. If screening programs continue to expand by adding a new screening test for each new disorder, it is obvious that expansion will continue to be slow and costly.

In a 1996 editorial in Clinical Chemistry, Sweetman noted that a significant technologic advance had occurred in the accurate measurement of amino acids and other compound important for the diagnosis of a larger group of genetic metabolic disorders. He referred specifically to tandem mass spectrometry, which he believed would likely have a significant impact in the sensitivity, specificity, and scope of newborn screening. Initially, manual fast atom bombardment tandem mass spectrometry was used to detect inborn errors in fatty acid, organic acid, and amino acid metabolism. The adaptation of this technique for use with dried blood specimens collected on filter-paper cards has made possible its use for newborn screening. More recently, electrospray tandem mass spectrometry has been shown to be an equally sensitive technique with the advantage of automation

The basis for tandem mass spectrometry analysis is the production of intact molecular species from a complex mixture by use of a soft ionization technique, followed by the identification of molecular fragments from specific components induced with neutral nitrogen or argon molecules. The use of two quadrupole mass spectrometers

linked in tandem and separated by a collision cell in which the secondary fragmentation takes place permits highly selective and specific analysis of various amino acids and acylcarnitines. Time-consuming chromatographic separations are unnecessary, because separation and analysis take place simultaneously and entirely within the instrument. This technology also permits the reduction of the detection limit to 1 nmol/mL of blood, and makes it possible to use dried filter-paper specimens.

The versatility if this method also permits analysis of a variety of different chemical compounds, including groups of α-amino acids and basic amino acids, simple by changing scanning parameters. Simultaneous screening thus becomes possible for a number of inborn errors of amino acid metabolism, including phenylketonuria. Before the development of tandem mass spectrometry, inborn errors of fatty acid oxidation and organic acid metabolism involving branched-chain amino acid catabolism were not detected easily in the neonatal period using filter-paper blood specimens. The tandem mass spectrometry technique is ideal for multidisorder screening because of its ability to rapidly identify a full spectrum of acylcarnitines derived from either intramitochondrial acyl-coenzyme(Co)compounds or from conjugates with free organic acids. Research results also should lead to the development of different scan functions to detect additional groups of compounds that could be useful for newborn screening.

MATERIALS AND METHODS

Filter-paper blood specimens were routinely collected as part of the supplemental newborn screening program in Pennsylvania and Ohio. Additional specimens were collected as part of a pilot screening program in North Carolina, and only for amino acids under contracts with the District of Columbia and several hospitals in Louisiana At present, our program is screening approximately 250,000 newborn annually. In addition to amino acid and acylcarnitine profiling, many of these newborn are also routinely screened for cystic fibrosis, congenital adrenal hyperplasia, galactosemia, biotinidase, deficiency and glucose-6-phosphate dehydrogenase deficiency as part of the supplemental newborn screening program. Most specimens are collected between age 1 and 3 days, and sent by mail or overnight courier to the centralized screening laboratory.

Preparation and derivatiaztion of the samples is carried out on single 3/16 -inch discs punched from filter-paper cards into the wells of microtiter plates. Internal standards consisting of a mixture of 2nmol each of a number of deuterated amino acids and acylcarnitines are added to each vial. Five hundred μl of methanol are added, and the vials are shaken for 30 minutes to elute the compounds of interest. The supernate is transferred to a second microtiter plate and evaporated to dryness under nitrogen. Butyl esters are formed by the addition of 60 μl of 3 mol/L HCl in n-butanol and incubation for 15minutes at 65℃. After a second evaporation under nitrogen, either 30 μl of a methanol/glycerol/octylsulfate mixture was added for fast atom bombardment ionization or more recently the sample is reconstituted in 100 μl of acetonitrile and water(1:1) for electrospray ionization.

Tandem mass spectrometry analysis in our laboratory currently uses four quadrupole tandem mass spectrometers, including a Sciex API 300, a Sciex API 365 (PE Sciex instruments, Concord, ON, Canada), and a Quattro II(Micromass, Inc, Danvers, MA). A cesium ion gun and a conventional insertion probe had been used for fast atom bombardment ionization. For electrospray ionization, we now use Perkin Elmer 200 injectors (Perkin-Elmer Corp, Norwalk, CT) and Shimadsu LC10AD high-performance liquid chromatography

pumps (Shimadsu, Kyoto, Japan) for the Sciex instruments and a Hewlett Packard 1050 autosampler and high-performance liquid chromatography injector (Hewlett Packard, Waldbrun, Germany) for the Quattro II instrument to deliver the sample via a continuous flowing stream of acetonitrile and water(1:1). Samples injected into this flowing solvent pass through the electrospray probe and onto source chamber. The probe has a sheath gas of zero air and a solvent flowing through a high-voltage capillary tip where the nebulization and ionization take place. As the droplets proceed into the high vacuum of the tandem mass spectrometer and encounter a drying gas (nitrogen), the solvent evaporates, and droplet size is reduced until only individual ions remain.

Amino acids share a common fragment. However, this fragment is a neutral molecule and not an ion. Neutral molecules, because they are uncharged, cannot be separated in the second mass analyzer. For butyl ester derivatives of α-amino acids, a neutral butyl formate forms by fragmentation in the collision cell. The mass of this molecule is 102. Since this conunon neural fragment molecule cannot be detected, a parent ion scan of mass 102 is not possible. However, the other fragment remaining after the loss of the butyl formate is indeed an ion. Hence, all product ions of α-amino acids will produce an ion that has a mass of 102 less than its parent molecular ion mass. To achieve selective detection using neutral loss scan functions, the mass acquisition in the second mass analyzer is linked and offset by a mass of 102 less than the mass being scanned in the first mass analyzer. Hence, if an ion in the first analyzer produces a neutral loss molecule of mass 102, the charged fragment ion will be 102 less than the product ion, and will be scanned by the second mass analyzer. These ions will pass through to the detector and the signal is recorded.

Acylcarnitines (as butyl ester derivatives), however, share a common fragment ion at mass 85. This fragment is part of the acylcarnitine backbone and is independent of the fatty acyl group attached to the carnitine molecule. For this reason, all acylcarnitines produce a mass 85 fragment ion. This is analogous to a selective filter, which allows only ions of mass 85 to pass through the second mass analyzer and reach the signal detector. With the first and second mass analyzer linked each time an ion event is recorded at the detector, the mass at the time this event occurred in the first mass analyzer is recorded. These ion events are recorded in a mass spectrum in which the x-axis represents the mass and the y-axis the number of ions striking the detector. Compounds that do not have a common fragment at mass 85 will not be detected even thought the mass was separated in the first mass analyzer region.

Another important advantage of tandem mass spectrometry is that these different scan function can be performed in series. For example, the first scan might be a neutral loss of mass 102, while the second scan is a parent ion scan of mass 85. In this manner, we have developed six different scan functions and interlaced their acquisition over a 1-minute period. We use these routinely in our program to measure α-amino acids; basic amino acids; free, esterified, and total carnitine; and acylcarnitines.

RESULTS

On November 1, 1992 we began routine screening of newborn filter-paper blood specimens with fast atom bombardment tandem mass spectrometry to detect inborn errors of amino acid-metabolism using limited amino acid profiling, as well as to detect inborn errors of fatty acid oxidation and branched-chain amino acid catabolism using acylcarnitine profiling. As of June 30, 1999 we had

screened 746, 337 newborns for amino acid disorders and 687,630 newborns for organic acid and fatty acid disorders. Table 1 summarizes the results of this screening program. A total of 163 inborn errors of metabolism were detected, including 86 in amino acid metabolism, 32 in organic acid metabolism, and 45 in fatty acid oxidation. The total frequency of disorders detected is approximately 1 in 4000 newborns screened. Fig, 1A shows an example of a scan of a newborn with a normal amino acid profile, and Fig. 1B, a newborn with phenylketonuria. Fig. 2A shows a normal newborn acylcarnitine profile, and Figure 2B, a profile from a newborn with medium chain acyl-coenzyme A (CoA) dehydrogenase deficiency.

Table 1. Results of Newborn Screening Using Tandem Mass Spectrometry

Disorder	No. of Cases
Amino Acid Disorders (746,337 newborns screened)	
Phenylketonuria	37
Hyperphenylalninemia	31
Maple syrup urine disease	9
Hypermethioninemia	4
Citrullinemia (acute neonatal)	3
Citrullinemia (mild)	1
Argininosuccinic aciduria	1
Organic Acid Disorders (687,630 newborns screened)	
Glutaric acidemia type 1	9
Methylmalonic acidemia (acute neonatal)	2
Methylmalonic acidemia (vitamin B12 responsive)	1
Methylmalonic acidemia (Maternal vitamin B12 deficient)	2
Propionic acidemia (acute neonatal)	2
Propionic acidemia (late onset)	3
Isovaleric acidemia (chronic)	1
3-hydroxy-3-methylglutaryl-CoA carboxylase deficiency	1
3-hydroxy-CoA carboxylase deficiency (maternal)	7
3-hydroxy-CoA carboxylase deficiency (isolated)	2
Fatty Acid Oxidation Disorders (601,440 newborns screened)	
Medium-chain acyl-CoA dehydrogenase deficiency	39
Very-long-chain acyl-CoA dehydroganase deficiency	3
Short-chain acyl-CoA dehydroganase deficiency	2
Multiple acyl-CoA dehydrogenase defieciency	1

CoA=coenzyme A.

DISCUSSION

Over the past 35 years newborns screening for inborn errors in amino acid metabolism (phenylketonuria, maple syrup urine disease, homocystinuria, tyrosinemia, etc) has evolved through several major technologic stages, including the use of Guthrie bacterial inhibition assays, paper and thin-layer chromatography, and fluorometric analysis. The introduction of automated electrospray tandem mass spectrometry has not only improved the sensitivity and specificity of the newborn screen, but has introduced the concept of a fully automated screening profile that detects multiple disorders at the same time. The earlier use of paper and thin-layer chromatography by some blood and urine screening programs in the 1960s and 1970s first introduced this concept, but the methods were not automated or quantitative, and were laborious and time consuming. The great sensitivity and specificity of tandem mass spectrometry can be appreciated when one reads the three validation articles by Chance et. al.

In a recent study with the California Department of Health Services, Chance et. al also compared the interpretation of fluorometric analytic results for phenylketonuria with tandem mass spectrometry in blood spots collected from newborns aged less than 24hours. They retrieved 208 blood spots that had been classified initially as negative, initially positive for hyperphenylalaninemia with negative results on recall, or initially positive with phenylketonuria or hyperphenylalan-

inemia confirmed on recall. The use of tandem mass spectrometry confirmed all previously detected cases, but more importantly it reduced the number of false positives from 91 to 3. The use of the phenylalanine-to-tyrosine ratio further reduced the false positives to 1. These results demonstrate the utility of tandem mass spectrometry for the routine screening of early-discharge newborns. With the cutoff values used in this study, we anticipated that few, if any, false negatives should occur, even with early discharge newborns. The establishment of specific cutoffs and the age at which newborn screening is determined to be reliable, however, rests with individual laboratories and screening programs. In our program, the false positive rate also gas been extremely low, and we are unaware of any false negative in more than 746,000 newborns screened.

Our experience with maple syrup urine disease screening also has been good. We have detected a number of affected newborns with specimens collected at age less than 24 hours. Although few of the newborns than we screen gave specimens collected this early, we have a unique situation with Mennonite newborns who have a high frequency of maple syrup urine disease. Because of the presence of a single common mutation in this inbred population, it has been possible to detect at-risk newborns through mutation screening of high-risk families. Dr. Holmes Morton in Lancaster Country does this routinely, and we receive blood specimens for branched-chain amino acid screening using tandem mass spectrometry on newborns aged less than 24 hours. The abnormal cases have been detected easily. The condition also has been detected in several non-Mennonite newborns.

Homocystinuria, however, presents a potential problem. When newborn screening for homocystinuria due to cystathione β-synthase deficiency was first developed in the late 1960s, the average age of the infant at hospital discharge was 5 to 7 days. Today the average age at discharge is 1 to 2 days. The rise in methionine in this condition is known to be slow, and concerns have been raised that specimens collected at age 1 to 2 days might not show an elevated level of methionine. The original cutoff for the bacterial inhibition assay for methionine was set at 12 mg/dl. In recent years some screening programs have lowered this to 1 mg/dl to avoid missing cases of homocystinuria. Because of the greater sensitivity of tandem mass spectrometry screening, we are able to further lower the cutoff to 0.7 mg/dl. we believe that this should be sufficiently sensitive to detect cases of homocystinuria at age 1 to 2 days. We cannot confirm this belief, however, because as yet we gave not detected any cases of homocystinuria in our program. We are also unaware of any missed cases. Cases of inborn error of the urea cycle, including acute citrullinemia and argininosuccinic aciduria, are also easily detectable.

While the introduction of automated tandem mass spectrometry has greatly improved the sensitivity, specificity, and scope of newborn screening for amino acid disorders, for the first time it also has made possible newborn screening for inborn errors in fatty acid oxidation and organic acid metabolism. We are confident of the ability of this methodology to detect accurately and reliably acute cases of methylmalonic, propionic, and isovaleric acidemias; glutaric acidemia type I; isolated 3-methylglurtaryl-CoA carboxylase and 3-hydroxy-3-methylcrotonyl-CoA carboxylase and 3-hydroxy-3-methylglutaryl-CoA lyase deficiencies; and medium-chain acyl-CoA dehydrogenase deficiency. We are not yet certain of the reliability of this method for the detection of all cases of methylmalonic acidemia (especially the cobalamin defects); the short-chain and very-long-chain acyl-CoA dehydrogenase deficiencies; and multiple acyl-CoA dehydrogenase deficiency. A number of other

disorders are theoretically detectable in the newborn period using tandem mass spectrometry, but as yet have not been detected in our newborn screening program. It is possible that some could have been missed in the early years of the program or they have been missed in the early years of the program or they might simply not have diagnostic metabolites present in the early newborn period. Other approaches or modifications in existing methodologic approaches might be required for detection.

The presence of false positives with acylcarnitine screening has not been a serious problem, with one possible exception: propionylcarnitine. An appropriate cutoff for propionylcarnitine has not been established because maternal and newborn cobalamin (bitamin B_{12}) deficiencies affect the levels of propionylcarnitine and because of the uncertainty of detecting inborn errors of cobalamin metabolism. The false positive rate for this metabolite has therefore been higher in our early studies while we have attempted to determine an appropriate cutoff that will not give false negatives for the milder forms of methylmalonic acidemia.

This exciting new methodology for newborn screening has been shown to be more specific and sensitive than of screening with the introduction of the concept of metabolic profiling. It also has been shown to be highly cost effective if screening is done in a large, regionalized screening program with the technical analytic chemistry and clinical biochemical genetic expertise required to maintain the high quality and standards necessary for success.

REFERENCES

1) Guthrie R, Susi A. A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. Pediatrics 1960;32:338-343.
2) Naylor EW. Recent developments in neonatal screening. Semin Perinatol 1986:9:232-249.
3) Desposito F, Cho S, Frias J, et al. Newborn screening fact sheets. Pediatrics 1996:98:473-501.
4) Sweetman L : Newborn screenig by tandem mass spectormetry (MS/MS), editorial. Clin Chem 1996; 42:345-346.
5) Millington DS, Norwood DL, Kodo N, et al. Application of fast atom bombardment with tandem mass spectrometry and liquid chromatography/mass spectrometry to the analysis of acylcarnitines in human urine, blood and tissue. Anal Biochem 1989;180:331-339.
6) Millington DS, Kodo N, Row 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;29:66-71.
7) Chane DH, Millington DS, Terada N, et al. Rapid diagnosis of phenylketonuria by quantitative analysis for phenylalanine and tyrosine in neonatal spots by tandem mass spectrometry Clin Chem 1993;39;66-71.
8) Chace DH, Hillman ST, Millington DS, et al. Rapid diagnosis of maple syrup urine disease with newborn blood spots by tandem mass spectrometry. Clin Cem 1995;41:62-68.
9) Chace DH, Hillman ST, Millington DS, et al. : Rapid diagnosis of homocystinuria and other hypermethionemias from newborn blood spots by tandem mass spectrometry. Clin Chem 1996;42:349-355.
10) Chace DH, Hillman SL, Van Hove JK, Naylor EW. Rapid diagnosis of MCAD deficiency. Quantitative analysis of octanoylcarnitine and other acylcrnitines by MS/MS. Clin Chem 1997;43:2106-2113.
11) Ziadeh R, Hoffman EP, Finegold DN, et al. Medium chain acyl-CoA dehydrogenase deficiency in Pennsylvania: Neonatal screening shows high incidence and unexpected mutation frequencies. Pediatr Res 1995;37:675-676.
12) Chace DH, Sherwin JE, Hillman SL, et al. 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 hours. Clin Chem 1998;44:2405-2409.
13) Rashed MS, Ozand PT, Bucknall DN, Little D: Diagnosis of inborn errors of metabolism from blood spots by acylcarnitines and aminoacids profiling using automated electrospray tandem mass spectrometry. Pediatr Res 1995;38:324-331.
14) Levy HL. Newborn screening by tandem mass spectrometry: New era, editorial. Clin Chem 1996; 44:2401-2402.