Identification of Infants at Risk for Developing Fabry, Pompe, or Mucopolysaccharidosis-I from Newborn Blood Spots by Tandem Mass Spectrometry

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Objective To assess the performance of a tandem mass spectrometry (MS/MS) technology in a newborn screening laboratory to simultaneously measure α-galactosidase, acid-α-glucosidase, and α-L-iduronidase for the detection of infants at risk to develop Fabry, Pompe, or mucopolysaccharidosis (MPS)-I diseases.

Study design Enzyme activity was assayed from a 3.2-mm punch from 100 000+ anonymous newborn blood spots. Punches with low enzyme activity were further evaluated by nucleotide sequence analysis of the responsible gene. Confirmation of affected infants was dependent on identification of mutations compatible with diminished enzyme activity.

Results The technology for simultaneously measuring multiple enzyme activities by MS/MS was successful. The confirmation of diagnosis for Fabry, Pompe, or MPS-I, by DNA sequencing estimated the prevalence of Fabry disease at 1/7800 males (95% CI 1/17 800-1/3600); Pompe disease at 1/27 800 newborns (95% CI 1/90 000-1/10 200); and MPS-I at 1/35 500 newborns (95% CI 1/143 000-1/11 100). These estimates of prevalence are 2 to 4 times greater than the prevalence estimated by clinical diagnosis. The combined prevalence for the 3 disorders was 1/7500 newborns (95% CI 1/13 500-1/4500).

Conclusions MS/MS for the simultaneous assay of multiple lysosomal enzymes can be successfully introduced into a routine newborn screening laboratory. The technology has a positive predictive value equal to, or better, than methods currently used for the detection of nonlysosomal disorders. Using newborn blood spots, the combined prevalence of Fabry, Pompe, and MPS-I is estimated at 1/7500 newborns based on low-enzyme activity and confirmation by mutation analysis. *(J Pediatr 2013;163:498-503)*.

More than 50 lysosomal storage diseases (LSDs), with an estimated clinical prevalence of 1/7000 to 1/9000 in European populations, have been described.[1,2] LSD symptoms and severity vary, from central nervous system degeneration in infancy in severe Krabbe disease or mucopolysaccharidosis (MPS)-I, to minor somatic complications in Gaucher disease, type 1. Each of the disorders, however, has a wide spectrum of clinical presentations depending on the effect mutations have on the residual enzyme activity. Even with the same mutation, differences in factors controlling gene expression and the environmental milieu will influence disease manifestation. Because of the variable clinical presentation and nonspecific symptoms of the LSDs, there is often delay in clinical recognition and diagnosis. This delay in diagnosis may postpone the timely initiation of therapy for the prevention of serious central nervous system or somatic involvement.[3]

Since the recognition by Chamoles et al[4,5] that lysosomal enzymes retain activity in dried blood spots on filter paper, our group has been designing novel substrates that can be used in diagnostic and newborn screening laboratories for the detection of LSD patients.[6] We have designed substrates and internal standards for the quantitative measurement of lysosomal enzyme activities by tandem mass spectrometry (MS/MS). These include methods for the detection of Gaucher (acid ß-glucosidase), Fabry (α-galactosidase [GLA] A), Pompe (acid α-glucosidase [GAA]), MPS-I (α-L-iduronidase [IDUA]), MPS-II (α-L-iduronide-2-sulfatase), Niemann-Pick diseases types A and B (acid sphingomyelinase), MPS-IVA (galactose-6-sulfate sulfatase), MPS-VI (N-acetylgalactosamine-4-sulfatase), and Krabbe (galactocerebrosidase) diseases.[7-13]

CDC	Centers for Disease Control and Prevention
GAA	α-glucosidase
GLA	α-galactosidase
IDUA	α-L-iduronidase
LSD	Lysosomal storage disease
MPS	Mucopolysaccharidosis
MS/MS	Tandem mass spectrometry
PCR	Polymerase chain reaction
QC	Quality control

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Many of these enzyme activity assays can be "multiplexed" by incubating samples with a cocktail containing substrates and internal standards in a common buffer, measuring the products by MS/MS,[14-18] and calculating enzyme activities. To evaluate the robustness of the MS/MS method, we tested a multiplexed assay for Fabry disease, Pompe disease, and MPS-I in the Washington State Newborn Screening Laboratory on over 100 000 anonymous newborn dried blood spots. These 3 disorders were selected on the belief that they would be clinically acceptable to the newborn screening community for early detection and intervention.

Methods

The screening of ~110 000 newborns was performed in the Washington State Newborn Screening Laboratory. Institutional Review Board approval was obtained from the Washington State Department of Health with the stipulation that the screening be done anonymously after all required newborn screens had been performed. Punches were obtained from blood spots that had been stored at 18°C for 8-10 months from collection.

Retention of 80% of initial enzyme activity was validated under these conditions. Two 3.2-mm punches were collected for each anonymous newborn screening sample. The first punch was used to determine enzyme activity. The second punch was used for sequence analysis for samples with enzyme activities in the affected range. MS/MS was used for quantitating enzyme activity for GLA A, acid GAA, and IDUA as reported by Duffey et al.[14]

Cocktails were prepared from vials containing substrate and internal standard from the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia.[19]

A 3.2-mm punch from each newborn dried blood spot was placed into a well of a 96-deep-well plate, and 30 μL of ammonium formate buffer (0.1 M, pH 4.4) containing 0.48 mM IDUA substrate, 3.1 μM IDUA internal standard, 0.2 mM GAA substrate, 2.0 μM GAA internal standard, 0.6 mM GLA substrate, 1.2 μM GLA internal standard, and 8 μM acarbose (Sigma Aldrich, St Louis, Missouri) was added. In addition to the newborn dried blood spots, each 96-deep-well plate contained 6 wells with a blank filter paper punch and 2 wells each of the 3.2-mm punches from quality control (QC) dried blood spots from the CDC.[15] The blanks and QC samples were in the first and last columns of each plate. All pipetting was performed using a Rainen Liquidator 96-tip pipetter. The plates were sealed with aluminum plate sealing film (VWR International LLC, Radnor, Pennsylvania) and incubated at 37°C for 16 hours (overnight) in an orbital, shaking incubator at 225 rpm.

In the morning, the plates were quenched by the addition of 100 μL of ammonium acetate buffer (0.1 M, pH 5.5), and 400 μL ethyl acetate was added to the wells and mixed by aspirating the liquid 20-40 times using the Rainen Liquidator (Rainen Instrument LLC, Oakland, California). The plates were covered with foil, centrifuged for 5 minutes at 3000 g, and 200 μL of the top ethyl acetate layer was transferred to a new 96-shallow-well plate. The ethyl acetate was evaporated in a stream of air with an SPE Dry 96 Dual Argonaut sample concentrator system (Biotage LLC, Charlotte, North Carolina), with a flow rate of 40-80 psi of air and heating <35°C (typically <30 minutes), and the residue was resuspended in 100 μL of the mass spectrometry mobile phase (80% acetonitrile, 20% water, 0.2% formic acid). The plates were placed on an orbital shaker for 5 minutes, covered with aluminum foil, and placed into the injector tray for MS/MS analysis.

A Waters Acquity TQD Ultra Performance (Waters Corporation, Milford, Massachusetts) tandem quadrupole MS/MS was used for analysis. Ten microliter of each 100 μL sample was injected using a Waters 2777C sample manager via flow injection. The injection fluid was 80/20 acetonitrile/water with 0.2% formic acid at 0.10 mL/min for 1.10 minutes and then 0.5 mL/min for 0.40 minutes. The enzyme activity was calculated from the abundance ratio of product to internal standard with subtraction of the average of the 6 blanks. Enzyme activity was reported in μmol/h/L of blood, assuming each punch contained 3.2 μL of blood.

For samples with enzyme activities in the affected range, genotype was determined by analysis of the nucleotide sequence of the appropriate gene. Genomic DNA was isolated from the duplicate 3.2-mm punch using a QIAamp DNA micro kit (Qiagen, Carlsbad, California). The Vector NTI Advance 11 computer program from Invitrogen (Carlsbad, California) was used to develop primers for amplification. Exons and the flanking intron boundaries of the *GAA* and *GLA* genes were amplified by polymerase chain reaction (PCR) using *Tsg* DNA Plus Polymerase (D102; Lamda Biotech, St. Louis, Missouri) following the supplier's instruction. The exons and the flanking introns of *IDUA* were amplified using the GC-RICH PCR system (#12140306001; Roche Applied Science, Mannheim, Germany) following the supplier's instructions. The PCR amplicons were sequenced using a 3130 Genetic Analyzer (Applied Biosystems, Foster City, California). Unless otherwise noted, primers used for PCR and sequencing reactions were identical. The Mutation Surveyor DNA Variant Analysis software from SoftGenetics (State College, Pennsylvania) was used to confirm sequencing genotypes. GenBank entries NM_000152.3 (*GAA*), NM_000169.2 (*GLA*), and NM_000203.3 (*IDUA*) were used as references for coding regions, whereby nucleotide A of the translation initiation codon ATG constituted numbering +1 of the complementary DNA sequences. We followed the standard naming convention of the Human Genome Variation Society such that methionine encoded by the translation mutation codon was designated as position 1 in amino acid numbering.

Dried blood spots used for QC were obtained from the CDC.[19] Four different samples were obtained: (1) unprocessed cord blood (high QC); (2) 50% cord blood in leukocyte-reduced base pool (medium QC); (3) 5% cord blood in leukocyte-reduced base pool (low QC); and (4) leukocyte-reduced base pool (blank). Two 3.2-mm punches from each QC blood spot were included in each 96-well assay plate. A continuous 95% CI was calculated for each QC