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Newborn Screening for Pompe Disease by Measuring Acid α-Glucosidase Activity Using Tandem Mass Spectrometry

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BACKGROUND: Pompe disease, caused by the deficiency of acid α-glucosidase (GAA), is a lysosomal storage disorder that manifests itself in its most severe form within the first months of life. Early detection by newborn screening is warranted, since prompt initiation of enzyme replacement therapy may improve morbidity and mortality. We evaluated a tandem mass spectrometry (MS/MS) method to measure GAA activity for newborn screening for Pompe disease.

METHODS: We incubated 3.2-mm punches from dried blood spots (DBS) for 22 h with the substrate [7-benzoylamino-heptyl)-{2-[4-(3,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-phenylcarbamoyl]-ethyl}-carbamic acid tert-butyl ester] and internal standard [7-d_5-benzoylamino-heptyl)-[2-(4-hydroxyphenylcarbamoyl)-ethyl]-carbamic acid tertbutyl ester]. We quantified the resulting product and internal standard using MS/MS. We assessed inter- and intrarun imprecision, carryover, stability, and correlation between enzyme activities and hematocrit and punch location and generated a Pompe disease–specific cutoff value using routine newborn screening samples.

RESULTS: GAA activities in DBS from 29 known Pompe patients were <2 μmol/h/L. GAA activities in routine newborn screening samples were [mean (SD)] 14.7 (7.2) μmol/h/L (n = 10 279, median 13.3, 95% CI 14.46–14.74 μmol/h/L) and in normal adult samples 9.3 (3.3) μmol/h/L (n = 229, median 9, 95% CI 8.88–9.72 μmol/h/L). GAA activity was stable for 28 days between 37 °C and −80 °C. Carryover could not be observed, whereas intrarun and interrun imprecision were <10%. The limit of detection was 0.26 μmol/h/L and limit of quantification 0.35 μmol/h/L.

CONCLUSIONS: The measurement of GAA activities in dry blood spots using MS/MS is suitable for high-throughput analysis and newborn screening for Pompe disease.

Pompe disease (OMIM 232300, glycogen storage disease II), which is caused by a deficiency of acid α-glucosidase (GAA),[6] is an autosomal recessive lysosomal storage disorder resulting in the progressive accumulation of glycogen, primarily in muscle tissue *(1)*. Patients with Pompe disease exhibit a wide spectrum of phenotypes, with the early infantile presentation at the most severe end of the disease continuum. Affected infants typically die within the first year of life from progressive cardiomyopathy, muscular hypotonia, and failure to thrive. The incidence of Pompe disease is estimated to be 1:40 000 but may vary in different ethnic populations *(1)*.

Recently, enzyme replacement therapy (ERT) using recombinant human GAA (rhGAA) from Chinese hamster ovary cells has become available *(2, 3)*. Results from clinical trials in patients with infantile-onset Pompe disease are encouraging, with improved cardiac, motor, and respiratory function following ERT and reduced mortality compared with historical Pompe disease controls *(2–4)*. ERT in these trials was initiated when clinical symptoms were already present, however, and not all symptoms were reversible *(3)*. ERT may be even more beneficial in individuals with Pompe disease when started at an asymptomatic stage after diagnosis in the newborn period *(3)*.

Until recently, the diagnosis of Pompe disease required the analysis of GAA enzyme activity in fibroblasts cultured from a skin biopsy and/or tissue from a

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[6] Nonstandard abbreviations: GAA, acid α-glucosidase; ERT, enzyme replacement therapy; DBS, dried blood spots; MS/MS, tandem mass spectrometry.

muscle biopsy because blood contains maltase glucoamylase, an α-glucosidase whose activity masks the deficiency of GAA and causes false-negative test results *(1)*. However, obtaining biopsy material is relatively invasive and time-consuming, and the use of muscle biopsies is also known to yield false-negative test results. Methods were developed to measure GAA in dried blood spots (DBS) using maltose *(5)* and acarbose *(6)* as inhibitors of maltase glucoamylase with the fluorogenic substrate 4-methylumbelliferyl-α-D-glucopyranoside (4-MUG) and a novel substrate, respectively. A Pompe disease newborn screening pilot program in Taiwan used acarbose and 4-MUG to measure GAA activity in DBS *(7)*.

The purpose of this study was to establish the utility of a previously reported tandem mass spectrometry (MS/MS) method to measure GAA activity *(6)* for Pompe disease screening in a newborn screening laboratory setting.

The protocol has been developed by Zhang and coworkers *(8)*.

Materials and Methods

SAMPLES

We used 10 279 anonymous DBS from infants born in Austria to generate a newborn reference range. Samples were included from term and preterm infants, and blood sampling typically occurred between 48 and 72 h of life. All samples were analyzed within 2 weeks of sampling and were shipped and stored at room temperature.

DBS from 229 anonymous adults that had been submitted to the laboratory for α-galactosidase analysis were used to generate an adult reference range. Patient samples (14 infantile-onset and 15 late-onset patients) were provided by Genzyme Corp. and by the Metabolic Outpatient Clinic at the University Children's Hospital Vienna. All samples were collected after informed consent and were stored desiccated at −80 °C. Samples from infantile-onset Pompe disease patients were collected during the first month of life; other samples were obtained during adolescence or adulthood. None of the patients were on ERT. The diagnosis of Pompe disease was previously confirmed in all patients. All studies were performed in compliance with the Declaration of Helsinki ethical principles for medical research involving human subjects. Written informed consent was obtained from all patients, their parents, or their caretakers before blood draw.

ENZYME ASSAY USING 3.2-MM DBS

Reagents. Samples were extracted in 20 mmol/L sodium-phosphate (Sigma) solution (pH 7.1). GAA assay buffer contained 0.34 mol/L sodium phosphate and 0.17 mol/L citrate buffer (Fluka), pH 4.0. We prepared GAA assay cocktail by the sequential addition of 1.8 mL of 100 g/L CHAPS (Sigma), 15.9 mL GAA assay buffer, and 0.3 mL of 0.8 mmol/L acarbose in water (Toronto Research Chemicals) to a vial containing 0.12 μmol internal standard [7-d_5-benzoylamino-heptyl)-[2-(4-hydroxy-phenylcarbamoyl)-ethyl]-carbamic acid tert-butyl ester] and 12 μmol GAA substrate [7-benzo ylamino-heptyl)-{2-[4-(3,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-phenylcarbamoyl]-ethyl}-carbamic acid tert-butyl ester]. Substrate and internal standard were given to us by Genzyme. The vial was vortex-mixed after each addition. The final GAA assay cocktail contained 0.67 mmol/L substrate, 6.6 μmol/L internal standard, 0.013 mmol/L acarbose, and 10 g/L CHAPS. We used Brand pipettors and Eppendorf single-channel pipettes for reagent preparation.

Sample preparation. We punched 3.2-mm DBS into 96-well plates (Greiner Bio-One) using a Wallac DBS Puncher (PerkinElmer). The first and last well of each plate contained blank filter paper. We added 70 μL extraction buffer to each well and sealed the plates with a silicone plate-sealer (Pall). Samples were extracted at 37 °C and 750 rpm with shaking for 1 h in a Wallac NCS Incubator (PerkinElmer). We transferred 10 μL extract to a new plate with 15 μL GAA assay cocktail in each well. The plates were sealed and incubated for 22 h at 37 °C. Enzyme reactions were quenched by adding 100 μL of 1:1 ethylacetate:methanol (Merck). After mixing, we transferred samples to a 96-well deep-well plate (Brand). To prepare the liquid-liquid extraction, we added 400 μL of 1:1 ethylacetate:methanol to each well, followed by 400 μL ethylacetate and 400 μL distilled water (B. Braun, Melsungen, Germany). Samples were mixed by pipetting up and down and centrifuged at 1400*g* for 4 min. We transferred 300 μL of the upper phase to a new plate using the Quadra3 pipetting machine (Tomtec) and dried it using the Minivap (Porvair Sciences). Samples were reconstituted in 100 μL of 80:20 acetonitrile:water (Fischer Scientific; Merck). All transfers were made using a Transferpette 12-channel pipette (Brand).

Tandem mass spectrometry. Analyses used a API 2000 triple-quadrupole mass spectrometer (PE Sciex), Neogram (PerkinElmer Wallac), and Analyst v1.1 software (MDS Sciex) in positive ion mode. Instrument settings were as follows: curtain gas pressure 20 Torr, collision cell pressure 4 Torr; ion spray voltage 5500V, source temperature 2500 °C; gas 1 10 psi; gas 2 70 psi; declustering potential 16V, focusing potential 380V; entrance potential 5V, collision energy 21V; collision exit potential 10V. Samples (10 μL) were injected into the electrospray source with a Gilson 819 Injection Module

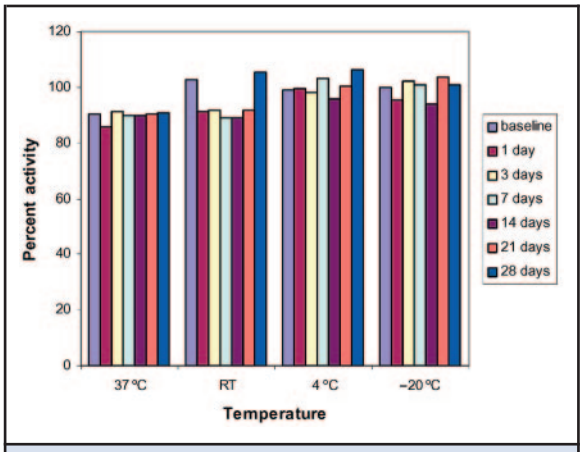

Fig. 1. Stability of enzyme activities.
Activities are shown as percentage of GAA activities at −80 °C.

(Gilson) using acetonitrile/water (80/20 vol/vol) as solvent at a flow rate of 250 μL/m (PerkinElmer Series 200 MicroPump). Analyses of GAA internal standard and product used multiple-reaction monitoring mode. Mass spectrometer parameters were optimized to give highest sensitivity for transitions m/z 503 > 403 (GAA internal standard) and m/z 498 > 398 (GAA product).

Results

ASSAY CHARACTERISTICS

GAA activity in DBS was stable at different temperatures ranging from −80 °C to 37 °C for up to 28 days. We measured GAA activities in 6 different punches from each card (mean activity at baseline: 15.58 μmol/h/L) and repeated measurements after 1, 3, 7, 14, 21, and 28 days of storage at the respective temperatures. No significant difference was found when each individual activity was compared to the baseline activity at −80 °C, although there was a trend to lower GAA activities when stored at room temperature and at 37 °C (Fig. 1).

We used the same samples to study the effect of hematocrit and location of the punch on GAA activity. There was no significant effect of the punch position on GAA activity, comparing central with peripheral punches (Fig. 2A). GAA activity showed a linear correlation with hematocrit values in the dried blood spots (R^2 = 0.9892 peripheral punches, R^2 = 0.9925 central punches) (Fig. 2B).

We measured activities in 12 punches from the same DBS to calculate intraassay variability. To examine interassay variability, we measured activities in 4 punches from different DBS on 7 days. Intraassay and

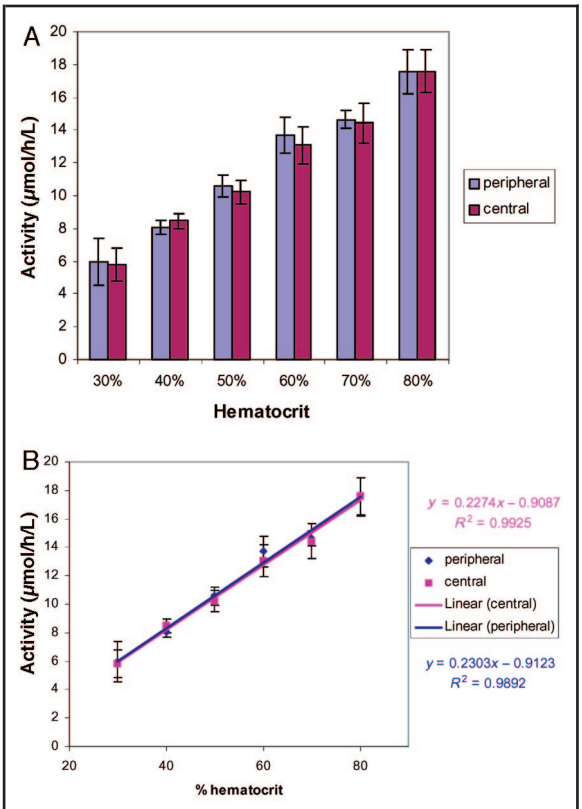

Fig. 2. Correlation between enzyme activity and hematocrit (A) and between enzyme activity and punch location (B).

interassay variability was <10% (Table 1). The limit of detection was 0.26 μmol/h/L, and the limit of quantification was 0.35 μmol/h/L. The mean carryover was −0.01 μmol/h/L (Fig. 3).

CONTROL AND PATIENT ACTIVITIES

GAA activities of the 10 279 newborn samples were normally distributed [mean (SD) 14.7 (7.2) μmol/h/L; median 13.3 μmol/h/L) (Fig. 4). GAA activities in 29 patients with Pompe disease were <2 μmol/h/L (Fig. 5).

Table 1. Inter- and intraassay imprecision (n = 6).

Variability	Average activity	SD	CV, %
Interassay	17.8	1.1	6.2
Punch 1	17.8	0.9	5.0
Punch 2	18.0	1.0	5.5
Punch 3	17.2	1.4	8.1
Punch 4	18.1	1.0	5.5
Intraassay	18.3	0.8	4.4

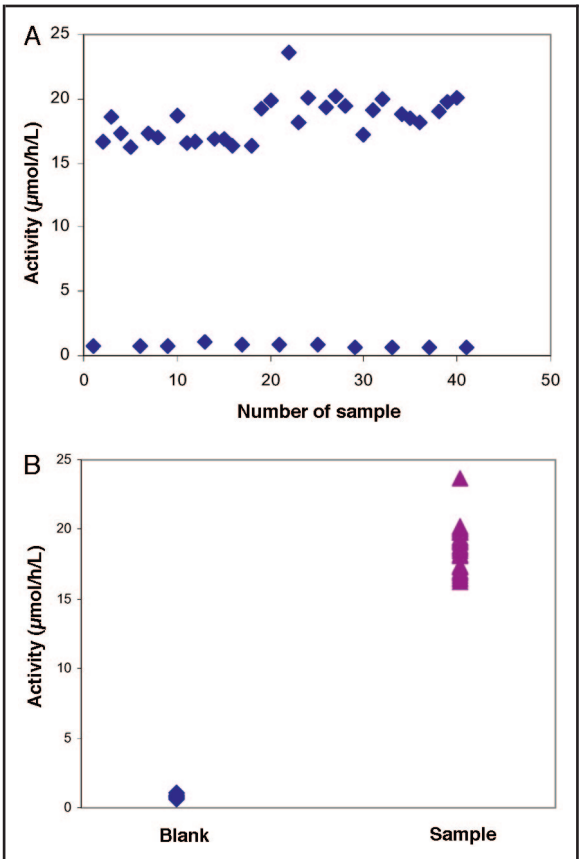

Fig. 3. Carryover between DBS and blank samples.
(A), Order of GAA analysis to evaluate carryover; (B), comparison between GAA activities in blank samples and DBS.

Samples from patients with infantile-onset disease and late-onset disease could not be differentiated based on enzyme activity. Only 4 of 10 279 newborn infants had a GAA activity <2 μmol/h/L (recall 0.039%). GAA activities in adults were 9.3 (3.3) μmol/h/L, median 9.

Discussion

Early diagnosis of Pompe disease may be facilitated through newborn screening, provided suitable analytical techniques are available for enzyme analysis in DBS. In addition, such techniques should demonstrate high-throughput capabilities, with acceptable diagnostic specificity and sensitivity. The late Nestor Chamoles and coworkers championed the use of a fluorogenic substrate 4-methylumbelliferyl glucose for analysis of GAA and other lysosomal enzymes in DBS *(5)*. They were able to demonstrate that the low GAA activities in patients with Pompe disease were clearly separated from obligate heterozygotes that had intermediate GAA activities and healthy controls in the presence of an inhibitor of the isoenzyme maltase-glucoamylase *(5)*. Additional techniques for newborn screening for lysosomal storage disorders including Pompe disease have been reported but have not been tested in a newborn screening setting *(8, 9)*. In particular, methods based on analyzing protein abundance with an ELISA-based method may miss mild cases of Pompe disease, as GAA may be functionally impaired but be produced at near-normal enzyme concentrations.

We chose to adapt and validate a recent analytical approach *(8, 11)* using tandem mass spectrometry for the measurement of GAA in dried blood spots for several reasons. First, MS/MS is readily available in most newborn screening laboratories. Second, MS/MS has demonstrated high-throughput capabilities. Third, MS/MS has low limits of detection and is specific for many analytes over a wide *m/z* range. Most importantly, measurement of GAA activity for the diagnosis of Pompe disease using MS/MS may be multiplexed with assays for diagnosis of Fabry disease, Krabbe disease, Niemann Pick disease types A and B, and Gaucher disease *(6, 8, 11)*.

The MS/MS assay for measurement of GAA activity showed intra- and interassay imprecision of <10%, with a mean carryover between samples of −0.01 μmol/h/L. In addition, GAA activity was independent of the location of the punch within the DBS, which has not been the case for some acylcarnitine species and amino acids *(12)*. GAA activities were linearly correlated with hematocrit values, indicating that most of the GAA activity is derived from cells and that hemoglobin does not interfere in the assay.

It is important for newborn screening to have stable analyte concentrations in DBS while samples are transported from the birth location to the screening center. Air temperatures and humidity may vary considerably between seasons. In our study, GAA activity was stable for up to 28 days at different temperatures, with only minor losses of activity when stored at room temperature or 37 °C for extended periods. Although we did not study the effect of humidity, it seems unlikely that humidity will have any significant effects on enzyme activities during transport to the screening laboratory.

GAA activity in more than 10 000 Austrian newborn infants, including preterm and term infants, showed a normal distribution, with a median activity of 13.3 μmol/h/L. All patients with Pompe disease had GAA activities of <2 μmol/h/L. Although infantile-onset Pompe disease patients tended to have lower GAA activities than adult Pompe disease patients, a clear separation of phenotypes was not possible based on enzyme activity in DBS, which is true for most other tissues *(13)*. Importantly, only 4 of 10 298 newborn

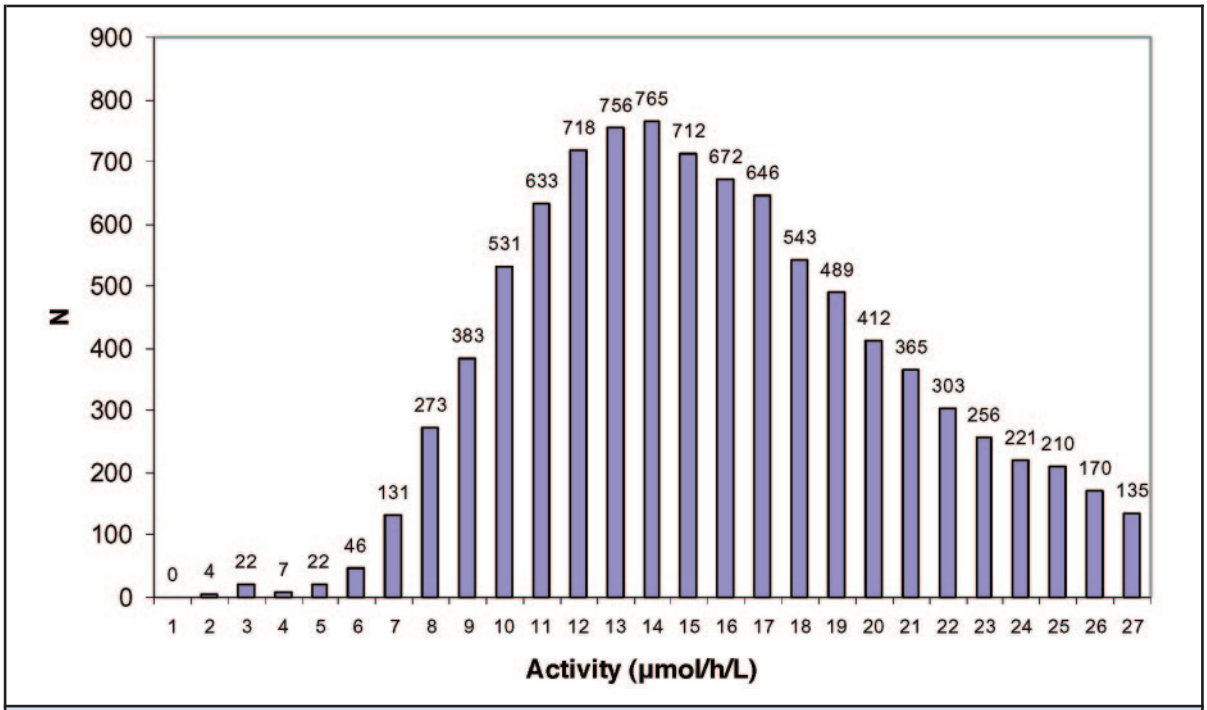

Fig. 4. α-Glucosidase activity in anonymous newborn screening filter cards (n = 10 279).

infants would have been recalled. This recall rate of 0.039% compares very favorably to other disorders in expanded newborn screening programs *(14)*. We confirmed the findings of Li et al. *(6)*, who were able to separate DBS of Pompe patients from those of controls, albeit at smaller numbers.

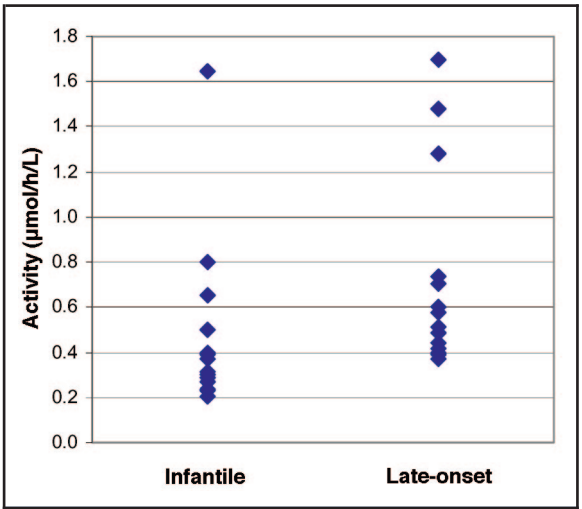

Fig. 5. α-Glucosidase activity in Pompe patients (n = 29).

We believe that the recall rate of 0.039% may be further reduced when molecular testing of the *GAA* gene in dried blood spots is added as second-tier testing. The goal of a Pompe disease newborn screening program is the identification of infantile-onset Pompe disease. Although most infantile-onset cases show clinical and laboratory signs, such as muscular hypotonia, cardiomyopathy, and increased CK activities, shortly after birth, there may be cases where diagnosis of infantile-onset disease may be equivocal. In addition, cases with late-onset disease will be detected, while clinical symptoms may not appear until adulthood. For these patients, the benefit of a newborn screening program is not evident, but may help to avoid significant delay in diagnosis and invasive diagnostic procedures and avert long-term sequelae.

We dedicate this work to the late Dr. Nestor Chamoles, a pioneer who laid the foundation for Pompe newborn screening.

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