Immunooquantification of α-Galactosidase: Evaluation for the Diagnosis of Fabry Disease

M aria Fuller,1,2* M elanie Lovejoy,1 D oug A. Brooks,1,2 M iriam L. Harkin,1 J ohn J. Hopwood,1,2 and P eter J. Meikle1,2

Background: Fabry disease is an X-linked inborn error of glycosphingolipid catabolism resulting from a deficiency of the lysosomal exoglycohydrolase, α-galactosidase. Enzyme replacement therapy is currently available for Fabry disease, but early diagnosis before the onset of irreversible pathology will be mandatory for successful treatment. Presymptomatic detection would be possible through the use of a newborn-screening program. We report on the use of sensitive assays for the measurement of α-galactosidase protein and activity and for the protein saposin C, which are diagnostic markers for Fabry disease.

Methods: Two sensitive immunoassays for the measurement of α-galactosidase activity and protein were used to determine the concentrations of α-galactosidase in dried filter-paper blood spots and plasma samples from control patients and patients with lysosomal storage disorder (LSD).

Results: Fabry hemizygous individuals were clearly identified from control populations by decreases in both α-galactosidase activity and protein. Fabry heterozygotes generally fell between the hemizyogotes and controls. Including the measurement of saposin C enabled differentiation between Fabry heterozygotes and controls. In blood spots, all Fabry individuals could be distinguished from control blood spots as well as from 16 other LSD patients.

Conclusions: The determination of α-galactosidase activity or protein in dried filter-paper blood spots could be used for the diagnosis of Fabry patients. With further validation, these assays could be used for the identification of Fabry patients in newborn-screening programs and may also be suitable for screening high-risk populations.

© 2004 American Association for Clinical Chemistry

Fabry disease is an X-linked lysosomal storage disorder (LSD) arising from a deficiency of the enzyme α-galactosidase, which cleaves terminal α-galactosyl moieties from various glycoconjugates (1, 2). Affected individuals accumulate glycosphingolipids, predominantly ceramide trihexoside, in the plasma and cellular lysosomes throughout the body. The classic phenotype manifests in childhood or early adolescence and is characterized by acroparasthesias, angiokeratoma, and corneal dystrophy. Progressive glycosphingolipid deposition leads to cardiovascular, cerebrovascular, and renal disease, which ultimately culminate in death in the fourth or fifth decade of life. In contrast to the classic phenotype, “cardiac” and “renal” variants have been identified, which present later in life with predominantly cardiac and renal disease, respectively, and lack the major classic symptoms of the disease (3, 4). Fabry disease most often affects males, although clinical manifestations in female heterozygotes can range from asymptomatic to a disease as severe as that suffered by hemizygous affected males (5).

α-Galactosidase (EC 3.2.1.22) is synthesized as a precursor protein with a molecular mass of 50 kDa, which is processed via a 47- to 50-kDa intermediate to the mature lysosomal form of 46 kDa (6). The carbohydrate moieties of the N-linked oligosaccharides of the lysosomal and secreted forms of recombinant human α-galactosidase (rha-gal) have been determined (7). The secreted form is heterogeneous, with complex and hybrid high mannose oligosaccharide structures as opposed to the lysosomal form, which only has a small amount of the complex high mannose oligosaccharide structures. Presumably the heterogeneous nature of the secreted form is attributable to

*Nonstandard abbreviations: LSD, lysosomal storage disorder; rha-gal, recombinant human α-galactosidase; and QC, quality control.
high-level production and differential glycosylation in the Golgi, whereas the intracellular form is generated by carbohydrate trimming in the lysosome.

Enzyme replacement therapy has recently become available for Fabry disease and has been shown to reduce lipid depositions in tissue biopsies (8–10). Nonetheless, for therapy to achieve a good long-term outcome, early diagnosis and treatment will be prerequisites. In the absence of a family member with a previous diagnosis of Fabry disease, the only logistical method to achieve early diagnosis is through a newborn-screening program. Following clinical suspicion, most often from the characteristic skin lesions and corneal dystrophy, diagnosis is confirmed by decreased α-galactosidase activity in blood and tissue samples. Classically, affected males usually have no detectable α-galactosidase activity, and concentrations in atypical hemizygous Fabry patients range from 5% to 35% of normal for a synthetic substrate (11).

To address the need for early diagnosis of Fabry patients, we have developed two sensitive and specific immunoassays to quantify α-galactosidase activity and protein concentrations in both dried filter-paper blood spots and plasma. We have used these assays to determine the concentration of α-galactosidase activity and protein in samples from Fabry hemizygotes, heterozygotes, apparently healthy controls, and other LSD patients. We have previously evaluated the use of two protein markers, LAMP-1 and saposin C, for the identification of individuals with a LSD at birth (12). Neither of these markers was able to differentiate LSD individuals from the control population, but saposin C was shown to be increased in Fabry disease. Therefore, in this report we also include immunoquantification of saposin C (13).

Materials and Methods

Patient Samples
The normal control dried filter-paper blood spots used in this study were deidentified and either obtained from the Neonatal Screening Laboratory in the Department of Genetic Medicine at the Women’s and Children’s Hospital, Adelaide, South Australia, or collected from adult blood donors by the Australian Red Cross Blood Bank in Adelaide, South Australia. Dried filter-paper blood spots were also obtained from previously diagnosed Fabry hemizygotes and heterozygotes.

The normal control plasma samples were from healthy volunteers, and the Fabry plasma samples were from patients and had been submitted to the National Referral Laboratory for the diagnosis of lysosomal, peroxisomal, and other genetic disorders in the Department of Genetic Medicine, Women’s and Children’s Hospital. Both dried filter-paper blood spots and plasma samples were stored at −20 °C.

Reagents
Genzyme provided purified rh-o-gal and the anti-α-galactosidase monoclonal antibodies 6F5.11.5.1 and 6G9.6.1.6. The monoclonal antibody 6F5.11.5.1 was labeled with Eu³⁺ chelate by use of the DELFIA® labeling reagents (Perkin-Elmer Life Sciences) and purified on a Pharmacia Superose 12 fast-phase liquid chromatography column as described previously (14). α-Galactosidase calibrators were prepared by diluting rh-o-gal in working buffer [0.1 mol/L citric acid, 0.2 mol/L Na₂HPO₄ (pH 5.5), 10 g/L bovine serum albumin, 2.5 g/L taurocholate, 2.5 mL/L Triton-X-100] or DELFIA assay buffer (Perkin-Elmer Life Sciences) to measure either α-galactosidase activity or protein, respectively. Quality-control (QC) material was prepared in 10 g/L ovalbumin in phosphate-buffered saline.

Immunoquantification of α-galactosidase Protein
Microtiter plates were coated with monoclonal antibody 6G9.6.1.6 (100 μL/well) at 5 mg/L in 0.1 mol/L NaHCO₃ for 16 h at 4 °C. The wells were washed twice with DELFIA wash buffer, and blood spots were placed in the wells with 100 μL of DELFIA assay buffer. For plasma and QC samples, 10 μL was diluted into 90 μL of assay buffer in each well. A nine-point calibration curve (0–500 pg/well) was included in each assay. The plates were shaken for 1 h at room temperature, incubated overnight at 4 °C, and then shaken for 1 h at room temperature the following day. The plates were washed six times with DELFIA wash buffer, and 100 μL of DELFIA assay buffer containing 200 μg/L of Eu³⁺-labeled monoclonal antibody (6F5.11.5.1) was added to each well. The plates were incubated at room temperature for 15 min with shaking and then overnight at 4 °C. The plates were then washed six times with DELFIA wash buffer, and 200 μL of DELFIA enhancement solution was then added to each well. The plates were incubated at room temperature for 10 min with shaking, and the fluorescence was read on a DELFIA 1234 Research Fluorometer. Concentrations of α-galactosidase were calculated from the calibration curve by use of Multicalc Data analysis software (Wallac 1234 software, Ver. 2).

Determination of α-galactosidase Activity
Microtiter plates were coated with 6G9.6.1.6, and blood spots, plasma, and QC samples were added to the coated wells as described above except that the wells were washed twice with 20 mmol/L Tris-HCl, 0.25 mol/L NaCl, pH 7.2. A calibration curve (0–500 pg/well) was included in each assay. The activity of rh-o-gal used for the calibration curve was determined with the fluorogenic substrate 4-methylumbelliferyl α-D-galactoside (11) before each assay, and the calibration curve was then expressed as activity per well. After the immunoassay, the plates were washed six times with 20 mmol/L Tris-HCl, 0.25 mol/L NaCl, pH 7.2, and 100 μL of 5 mmol/L 4-methylumbelliferyl α-D-galactoside was added per well. The microtiter plates were shaken for 5 min at room temperature and read on a DELFIA 1234 Research Fluorometer.

The fluorescence was then read on a DELFIA 1234 Research Fluorometer.
temperature and then incubated for 4 h at 37 °C. The enzyme reaction was stopped by the addition of 100 μL/well glycine buffer (200 mmol/L glycine, 158 mmol/L sodium bicarbonate, 146 mmol/L sodium hydroxide), pH 10.7. The fluorescence was read on a Wallac Victor2 1420 multilabel counter (Perkin-Elmer-Life Sciences), and α-galactosidase activity was calculated from the calibration curve by use of Multicalc Data analysis software.

SAPOSIN C
Immunooquantification of saposin C protein in blood spots was performed with a three-tiered assay as described previously (15). Saposin C concentrations were calculated by reference to a calibration curve (0–500 pg/well) run with each assay.

RESULTS
IMMUNOQUANTIFICATION OF α-GALACTOSIDASE PROTEIN CONCENTRATION AND ACTIVITY
The monoclonal antibody 6G9.6.1.6 captured >95% of rhα-gal as determined by assaying of the uncaptured protein and complete recovery of α-galactosidase protein and activity from whole blood to which it had been added (data not shown). Both immunoassays for α-galactosidase activity and protein concentration were optimized to yield a linear response over the biological range (Fig. 1). The detection limit for the α-galactosidase protein concentration assay was 2 pg/well (0.6 μg/L of whole blood and 0.2 μg/L of plasma) and for the α-galactosidase activity assay was 0.7 pmol·min⁻¹·well⁻¹ (0.02 μmol·min⁻¹·L⁻¹ of whole blood)⁻¹ and 0.007 μmol·min⁻¹·L⁻¹·(L of plasma)⁻¹. Either a control blood spot or 10 μL of control plasma was added to the calibrators, and no inhibition was observed for either blood spots or control human plasma for the α-galactosidase protein assay (Fig. 1A). For the α-galactosidase activity assay, no inhibition was observed for blood spots, but plasma did have a slight inhibitory effect (~10%) on the α-galactosidase activity detected (Fig. 1B).

Blood spots stored at −20 °C over an 8-month period and those stored at room temperature for 2 weeks showed no decrease in enzyme activity or protein concentration (data not shown). The stability of α-galactosidase in plasma was determined at 4 °C and −20 °C and after cycles of freezing/thawing over a period of 14 days. The amounts of α-galactosidase activity and protein detected in plasma decreased markedly over the 14-day period at 4 °C but remained unchanged at −20 °C. However, in plasma that had been thawed, both α-galactosidase activity and protein were decreased. Plasma that underwent repeated freeze/thaw cycles over the 14-day period showed further losses of α-galactosidase activity and protein.

To assess assay performance, we included low- and high-concentration QC samples in each run with interassay CVs <13% for protein (19 measurements over 100 days) and <19% for activity (18 measurements over 100 days). Intraassay CVs were <12% (n = 16) for the low and high QC materials in both assays. Intraassay CVs (n = 10) for adult normal control blood spots were 6% and 10% for α-galactosidase concentration and activity, respectively, and intraassay CVs (n = 10) for adult normal control plasma were 3% and 23%, respectively.

![Fig. 1](image-url)
α-galactosidase and saposin C in blood spots from adult and newborn controls, Fabry hemizygotes, and heterozygotes

Blood spots from newborn (n = 96) and adult (n = 145) normal controls were assayed for α-galactosidase protein and activity. The median concentration of α-galactosidase protein in the blood spots from newborns was 38.8 μg/L with 5th and 95th percentiles of 20.8 and 75.1 μg/L, respectively (Fig. 2A). The corresponding median enzyme activity for blood spots from newborns was 1.35 μmol·min⁻¹·L⁻¹, with 5th and 95th percentiles of 0.77 and 2.55 μmol·min⁻¹·L⁻¹, respectively (Fig. 2A). The median concentration of α-galactosidase protein in the blood spots from adults was 12.8 μg/L, with 5th and 95th percentiles of 6.7 and 26.3 μg/L, respectively (Fig. 2B). The corresponding median enzyme activity in the blood spots from adults was 0.542 μmol·min⁻¹·L⁻¹, with 5th and 95th percentiles of 0.28 and 0.99 μmol·min⁻¹·L⁻¹, respectively (Fig. 2B). We observed a significant correlation between α-galactosidase protein concentration and activity in newborn normal controls (Pearson correlation, 0.951; Fig. 2A). The correlation between α-galactosidase protein concentration and activity for adult normal controls was similar (Pearson correlation, 0.869; Fig. 2B).

The α-galactosidase and saposin C protein concentrations and α-galactosidase activity were measured in blood spots from 71 controls, 13 Fabry hemizygotes, and 4 heterozygotes (Fig. 3). All Fabry hemizygotes had α-galactosidase activities below the detection limit of the assay [0.02 μmol·min⁻¹·(L of whole blood)⁻¹] and could be distinguished clearly from the control population (Fig. 3A). Measurement of α-galactosidase protein also enabled differentiation of Fabry hemizygotes from controls (Fig. 3B). In both instances the Fabry heterozygotes fell between the Fabry hemizygotes and controls. Differentiation of the Fabry heterozygotes was achieved by including the measurement of saposin C. The ratio of saposin C to α-galactosidase protein could distinguish all four Fabry heterozygotes from the control group (Fig. 3C).

Fig. 2. Correlation of α-galactosidase protein and enzyme activity in blood spots from normal control newborns and adults.

Immunocaptured α-galactosidase protein and activity were determined in control blood spots from 96 newborn samples (A) and 145 adult samples (B). Results represent the amount of α-galactosidase detected per liter of whole blood.

α-galactosidase and saposin C in plasma from controls, Fabry hemizygotes, and heterozygotes

Plasma samples from normal controls (n = 50) Fabry hemizygotes (n = 28), and heterozygotes (n = 3) were assayed for α-galactosidase protein concentration and activity and for saposin C (Fig. 4). All Fabry hemizygotes, with the exception of one, had α-galactosidase activity below the detection limit of the assay [7 μmol·min⁻¹·(L of plasma)⁻¹]. As shown in panels A and B of Fig. 4, α-galactosidase activity and protein concentration enabled all Fabry hemizygotes and the three heterozygotes to be distinguished from controls. Unlike the blood spots, the ratio of saposin C to α-galactosidase protein provided no further differentiation of the heterozygotes from the control group (Fig. 4C) compared with measuring the α-galactosidase protein concentration alone.

α-galactosidase protein and activity in Fabry and other LSDs

The α-galactosidase protein concentration and activity were determined in blood spots from 145 controls, 12–13 Fabry hemizygotes, and 16 other LSDs (Fig. 5). There was no overlap between the control range and the Fabry hemizygotes for α-galactosidase protein (Fig. 5A). From the 52 other LSD patients measured for α-galactosidase protein, there was one mucopolysaccharidosis type IIIA patient who had protein concentrations in the range for Fabry patients. However, the measurement of α-galactosidase activity clearly identified all Fabry hemizygous patients from the controls and patients with other LSDs,
Fig. 3. α-Galactosidase protein and activity and saposin C concentrations in blood spots from Fabry hemizygotes, heterozygotes, and controls.

Blood spots from 71 controls, 13 Fabry hemizygotes (Hem), and 4 heterozygotes (Het) were assayed for α-galactosidase protein and activity and for saposin C, as described in the Materials and Methods. (A), α-galactosidase activity; (B), α-galactosidase protein; (C), ratio of α-galactosidase protein to saposin C. Bars inside boxes indicate the median value for each group; shaded areas indicate the 25th and 75th percentiles; error bars indicate the limits of the range. ○ and + represents statistical outliers and extreme statistical outliers, respectively. Results are expressed per liter of whole blood.

Fig. 4. α-Galactosidase protein and activity and saposin C in plasma from Fabry hemizygotes, heterozygotes, and controls.

Plasma from 50 controls, 26 Fabry hemizygotes (Hem), and 3 heterozygotes (Het) were assayed for α-galactosidase protein and activity, and saposin C, as described in Materials and Methods. (A), α-galactosidase activity; (B), α-galactosidase protein; (C), ratio of α-galactosidase protein to saposin C. Bars inside boxes indicate median value for each group; shaded areas indicate the 25th and 75th percentiles, and the error bars indicate the limits of the range. ○ and + represents statistical outliers and extreme statistical outliers, respectively. Results are expressed per liter of plasma.
Discussion

Because of the variability in clinical expression of Fabry disease, it is likely that this disorder is underdiagnosed, particularly the renal and cardiac variants, which often present later in life with limited symptoms. Renal insufficiency is a major clinical feature of Fabry disease, often progressing to end-stage renal failure requiring dialysis. It has been suggested that the prevalence of Fabry disease in large dialysis programs is underestimated (16). Population screening of high-risk groups such as those treated at renal dialysis and cardiac clinics may identify additional patients suffering from Fabry disease. With the availability of a recombinant form of α-galactosidase, it is now possible to treat Fabry disease by enzyme replacement (8). This introduces opportunities for early diagnosis to enable commencement of enzyme replacement therapy before the onset of clinical symptoms so that therapy can produce a good long-term outcome. Fabry patients rarely exhibit symptoms at birth; manifestations are most common in the second decade of life. Nonetheless, many patients, including heterozygous females, display symptoms of Fabry disease much later in life, and these individuals are often misdiagnosed (17). The only practical way to identify all Fabry individuals, as well as heterozygotes, is through screening programs.

The measurement of α-galactosidase for the diagnosis of Fabry disease is conventionally performed in leukocytes (11), but the use of dried filter-paper blood spots would be readily amenable to mass screening programs because of the simplicity of sample collection and transport. The applicability of blood spots for measurement of α-galactosidase activity has been reported (18). In this study we evaluated the measurement of α-galactosidase protein concentrations and activity in blood spots. We established sensitive immunoquantification methods for the measurement of the α-galactosidase protein concentration and activity in blood spots. We noted significant differences in both the protein concentration and enzyme activity. The newborn population had α-galactosidase concentrations and activities 2.5-fold higher than the adult population. There was also a wider range of protein concentration and enzyme activity in the newborn population compared with the adult population (Fig. 2). This is most likely a consequence of the greater numbers and broader range of leukocytes present in whole blood of newborns and has been observed for other lysosomal enzymes (19).

The use of α-galactosidase activity and protein concentration as a potential marker for diagnosis of Fabry disease enabled the identification of all hemizygotes (n = 12) from 145 controls (Fig. 3, A and B) and 52 blood spots from patients with other LSDs (Fig. 5). The Fabry heterozygotes (n = 4) could not be totally differentiated from the control group but generally had α-galactosidase protein concentrations and activities below or at the low end of the control range (Fig. 3, A and B). Including the measurement of saposin C and expressing this as a ratio to α-galactosidase protein enabled complete differentiation of the Fabry heterozygotes from the control group (Fig. 3C). Saposin C has been reported previously to be increased in blood spots from patients with Fabry disease (12). Further studies with greater numbers of heterozygous Fabry samples are needed to evaluate the applica-

---

Fig. 5. High-low plots of α-galactosidase protein and activity in blood spots from normal controls and LSD patients.

The α-galactosidase protein concentrations (A) and activity (B) were determined in blood spots from controls and LSD patients. The number of samples in each group is given in parentheses. □ indicates the mean value of each group, and error bars indicate the highest and lowest value for each group. GM1, GM1 gangliosidosis; MLD, metachromatic leukodystrophy; MPS, mucopolysaccharidosis; ML, mucolipidosis; NP, Niemann–Pick disease. Results are expressed per liter of whole blood.

Based on the absence of detectable activity in the Fabry blood spots (Fig. 5B).
bility of this approach for the detection of heterozygotes. Moreover, it may be useful to do this in conjunction with a clinical assessment to determine the relative severity of disease in Fabry heterozygotes.

None of the patients we evaluated in this study had decreased α-galactosidase activity with normal concentrations of α-galactosidase protein. Nevertheless, this is an issue for mass screening using α-galactosidase protein concentration alone because it has the potential to generate false-negative results. To fully discover the magnitude of false negatives, a larger number of Fabry samples need to be analyzed. It may then be worth including other screening markers, such as α-galactosidase activity and saposin C, to detect all Fabry individuals.

The integrity of α-galactosidase in blood spots is important when considering screening programs because samples may be collected in different areas and transported. We have shown that α-galactosidase activity and protein were stable in blood spots for at least 8 months when stored at −20 °C. On the other hand, plasma α-galactosidase activity and protein were shown to be relatively unstable (data not shown). An accurate measure of the immunocaptured α-galactosidase activity and protein concentration could be obtained only from fresh plasma samples. We also noted a rather high CV (23%) for protein concentration could be obtained only from fresh blood samples. We also noted a rather high CV (23%) for α-galactosidase activity in the plasma, which may be a consequence of its stability and therefore the accuracy of measurements. Further investigation into the stability of α-galactosidase in plasma is warranted if accurate measures of protein concentration and activity are to be determined. From a pragmatic point, blood spots are best suited for screening programs, compared with whole blood, plasma, or serum samples.

In conclusion, we have developed two methods for the determination of α-galactosidase activity and protein concentrations that are simple, rapid, and sensitive. This methodology has enabled the identification of Fabry hemizygotes on the basis of either enzyme activity or protein concentration in blood spots. Only four Fabry heterozygotes were included in this study, but when combined with the measurement of saposin C, the α-galactosidase assays differentiated all of them from the control population. A larger cohort of both newborn and adult control blood spots, Fabry hemizygotes, and heterozygotes will be required to validate these assays if they are to be used for mass screening. We have previously evaluated two proteins, LAMP-1 and saposin C, as markers for newborn screening, and although saposin C was significantly different in Fabry individuals compared with controls, identification of Fabry heterozygotes was not addressed (12). The determination of either α-galactosidase protein concentration or enzyme activity, along with saposin C, may be suitable for newborn screening for Fabry disease as well as screening of high-risk populations such as those at renal and cardiac clinics.

This work was supported by Genzyme Corporation (US) and in part by the NH&MRC (Australia). We gratefully acknowledge the Fabry patients and their families for providing blood spots and plasma samples, the South Australian Newborn Screening Centre for providing newborn blood spots, and the Australian Red Cross Blood Bank in Adelaide for providing control blood spots from adult blood donors.

References

