European Journal of Medical Genetics 58 (2015) 681–684

Contents lists available at ScienceDirect

European Journal of Medical Genetics

journal homepage: http://www.elsevier.com/locate/ejmg

Enzymatic diagnosis of Fabry disease using a fluorometric assay on dried blood spots: An alternative methodology

Eric Caudron [a], Patrice Prognon [a], Dominique P. Germain [b, *]

[a] Department of Pharmacy, AP-HP, Hôpital Européen Georges Pompidou, Paris, France
[b] University of Versailles – St Quentin en Yvelines (UVSQ), Division of Medical Genetics, Montigny, France

ARTICLE INFO

Article history:
Received 26 October 2013
Received in revised form
17 October 2015
Accepted 24 October 2015
Available online 28 October 2015

Keywords:
Fabry disease
Dried blood spot
Fluorometric assay
Zinc sulfate
Lysosomal diseases

ABSTRACT

Fabry disease (FD, OMIM#301500) is an X-linked lysosomal storage disorder caused by the functional deficiency of α-galactosidase A, a lysosomal enzyme. A method to screen for FD in large populations has been developed using a fluorometric assay of α-galactosidase A activity in dried blood spots (DBS) on filter paper. However, results can be influenced by quenching of fluorescence by haemoglobin which, together with small sample size, may result in a low light emission signal.

An alternative, simple and sensitive fluorometric assay was developed for the determination of α-galactosidase A activity in DBS. The assay uses 4-methylumbelliferyl-α-D-galactose as an artificial substrate. To minimize the risk of false-positives, zinc sulfate was used for protein precipitation to stop the enzymatic reaction and eliminate interfering species (hemoglobin). Samples from 209 individuals (60 hemizygotes, 68 heterozygotes, and 81 controls) were tested to establish reference values for the assay.

The mean α-galactosidase A activity of the 81 controls was 9.1 ± 3.3 μmol h^{-1} L^{-1} (mean ± SD). All 60 hemizygotes affected with FD had AGAL activities below 1.7 μmol h^{-1} L^{-1} (0.2 ± 0.3 μmol h^{-1} L^{-1}). For the 68 heterozygous females, AGAL activity ranged from 0 to 12.6 μmol h^{-1} L^{-1} (3.5 ± 2.7 μmol h^{-1} L^{-1}). Two-thirds of the female patients could be identified using the enzymatic assay and a cut-off level of 40% of the median control value (<3.4 μmol h^{-1} L^{-1}).

Our fluorometric assay using zinc sulfate protein precipitation was shown to have similar sensitivity and robustness while reducing the risk of false positive results due to quenching of 4-MU fluorescence by haemoglobin.

1. Introduction

Fabry disease (FD, OMIM#301500) is a devastating, progressive lysosomal storage disorder of glycosphingolipid metabolism. Absent or deficient activity of the exoglycohydrolase α-galactosidase A (α-D-galactoside galactohydrolase, EC 3.2.1.22; AGAL) (Germain, 2010) results in progressive lysosomal accumulation of neutral glycosphingolipids, primarily globotriaosylceramide (Gb_3 or GL-3), and related glycosphingolipids in a variety of cell types. Lysosomal and cellular dysfunction is believed to trigger a cascade of events including cellular death (podocytes and cardiomyocytes), inflammation, small vessel obstruction, and tissue ischemia. Development of irreversible cardiac and renal tissues fibrosis leads to major clinical events, such as stroke, cardiac hypertrophy, arrhythmia, and end-stage renal disease (Desnick et al., 2003). Delays between the onset of symptoms and diagnosis of FD commonly occur due to the lack of awareness of the disease, variability in clinical presentation, and non specificity of several symptoms. The advent of enzyme replacement therapy (ERT) has introduced the possibility of treating FD to prevent or slow down the progression to irreversible organ damage (Eng et al., 2001; Schiffmann et al., 2001; Germain et al., 2007, 2015) while clinical trials with pharmacological chaperons are currently ongoing (Germain et al., 2012). Early detection of the disease has therefore become critical. Newborn screening is the most practical approach to identify patients before the development of symptoms (Hwu et al., 2009). In addition, screening of patients in at-risk groups may unveil previously unrecognized FD patients exhibiting late-onset symptoms, which has important implications for therapeutic management (Linthorst et al., 2010), even if careful attention should be paid to the nature of the *GLA* variants identified during any screening protocol, since while some

* Corresponding author. Division of Medical Genetics, University of Versailles – St Quentin en Yvelines, 78180, Montigny, France.
E-mail address: dominique.germain@rpc.aphp.fr (D.P. Germain).

http://dx.doi.org/10.1016/j.ejmg.2015.10.014
1769-7212/

alleles are disease-causing and responsible for either the classic (e.g., p.R227*) or late-onset (e.g., p.F113L, p.N215S) phenotypes of Fabry disease, others are variants of unknown significance (VUS) or possible modifiers or genetic risk factors (e.g., p.R112H, p.R118C, p.A143T), and a third group includes non-pathogenic polymorphisms (e.g., p.E66Q, p.S126G, p.D313Y).

Leukocytes and plasma have been the most widely used biological specimens for the diagnosis of FD. Furthermore, measuring AGAL enzyme activity in leukocytes using 4-methylumbelliferyl-α-D-galactose as fluorogenic substrate has become the gold standard enzyme assay for diagnosing FD in males (Mayes et al., 1981). The use of leukocytes or plasma samples has, however, a number of disadvantages if used for screening purposes; it requires relatively large volumes of blood, subsequent laboratory processing, and proper storage, shipping and handling conditions. In order to overcome this drawback, a technique using dried blood spots (DBS) on filter paper suitable for large-scale screening for FD has been developed (Chamoles et al., 2001), and has since become a reference method. Dried blood samples require less biological material, and enzymatic activities are substantially more stable than in whole blood samples (*i.e.* up to several months at −20 °C or +4 °C) (De Jesus et al., 2009). The accuracy of AGAL activity assays using DBS has also been compared to leukocyte assays and the results suggest that DBS could be a useful diagnostic assay (Lukacs et al., 2007). However, the DBS method may be lacking reliability and robustness (Finsterer et al., 2004; Oemardien et al., 2011). Here we describe an adaptation of the original fluorometric assay (Chamoles et al., 2001) that uses DBS for the screening of FD.

2. Methods

EDTA blood samples were obtained from 60 hemizygous males and 68 heterozygous females affected with FD, and from 81 healthy individuals. The samples were spotted on filter paper (Schleicher and Schuell n° 903) and dried between 8 and 72 h at room temperature. Filter papers were then stored at +4 °C in plastic bags with desiccant until analysis. For each sample, a 3-mm diameter punched-out circle paper was placed into a 0.5 mL Eppendorf tube. The amount of blood per punch was estimated to be 3.4 μL (Mei et al., 2010). Fifty microliters of 0.1 mol L^{-1} acetate buffer (pH = 4.6), containing 4-methylumbelliferyl-α-D-galactopyranoside (5 mmol L^{-1}) as substrate and N-acetyl-D-galactosamine (75 mmol L^{-1}) as a specific α-galactosidase B inhibitor, were added. After gentle mixing, the tubes were incubated for 20 h at 37 °C in a water bath. The enzymatic reaction was stopped by the addition of 300 μL zinc hydroxide suspension (aqueous zinc sulfate heptahydrate (2%, w/v): 0.1 mol L^{-1} sodium hydroxide). After 5 min of gentle mixing to allow total protein precipitation, samples were centrifuged at 10,000 g for 10 min. Twenty microliters of 1 mol L^{-1} EDTA tetrasodic solution, adjusted at pH = 13 with NaOH 8 mol L^{-1}, were then added to 300 μL of the supernatant (final pH = 10.4) for fluorescence reading. All assays were performed in duplicate using two different punches of dried blood. The fluorescence (excitation filter, 355 nm and emission filter, 460 nm) of the 4-methylumbelliferone (4-MU) was measured on a Victor multilabel plate reader (Perkin–Elmer, Fremont, CA, USA). All measurements were corrected for fluorescence background, and the results were compared with the fluorescence from 4-MU standard curve. Enzymatic activities were expressed as micromoles of substrate hydrolyzed per hour and per liter of blood (μmol h^{-1} L^{-1}). The enzymatic activity of β-galactosidase, another lysosomal enzyme, was systematically assayed as a control enzyme to assess the quality of the biological samples. All samples were stored at +4 °C and all fluorometric enzyme activity assays were performed within 3 months after sampling.

In addition, we compared our modified assay with the original fluorometric AGAL assay in DBS (Chamoles et al., 2001) using samples from 20 patients affected with FD (8 hemizygotes, 12 heterozygotes) and 13 controls.

3. Results

A total of 209 subjects (128 patients, 81 controls) were enrolled in the study and the results of AGAL enzymatic activities are shown in Fig. 1. Results of the enzymatic activity of β-galactosidase in DBS were all normal, confirming sample integrity. A positive screening test for FD was defined as an AGAL activity below 3.4 μmol h^{-1} L^{-1}. This cut-off level represented 40% of the median control AGAL activity of 8.6 μmol h^{-1} L^{-1} (average ± SD = 9.1 ± 3.3 μmol h^{-1} L^{-1}) obtained for the 81 controls. Enzyme activities of the controls ranged from 4.2 to 17,3 μmol h^{-1} L^{-1}. By using 3.4 μmol h^{-1} L^{-1} as cut-off, no false-positive was detected among the normal subjects with the new zinc sulfate precipitation method. Therefore, the specificity [true negative/(true negative + false positive] of the method could be estimated at 81/(81 + 0) = 1. AGAL activities in the 60 hemizygotes affected with FD were all below 1.7 μmol h^{-1} L^{-1} (0.2 ± 0.3 μmol h^{-1} L^{-1}). Therefore, the sensitivity [true positive/(true positive + false negative)] of the screening test was estimated to be equal to 1 for male patients. For the 68 known heterozygotes, AGAL activity ranged from 0 to 12.6 μmol h^{-1} L^{-1} (3.5 ± 2.7 μmol h^{-1} L^{-1}). The sensitivity of the screening test in females was estimated at 45/(45 + 23) = 0.67. The comparison of the results obtained with our modified assay and the original fluorometric DBS assay (Chamoles et al., 2001) is presented in Fig. 2.

4. Discussion

The original dried blood spot method for the fluorimetric enzymatic assay of Fabry disease (Chamoles et al., 2001) can lead to important variations of the fluorescence reading (*i.e.* relative standard deviation ~20%) within the same sample, likely due to hemolysis of the dried blood sample. Indeed, a rather intensely colored solution after enzymatic reaction was observed when using the original method. The maximum absorbance was at 405 nm, a wavelength that strongly overlaps with Soret's band of the heme, a cyclic tetrapyrrole, and its by-products. More intense absorbance was associated with more pronounced fluorescence inhibition. Fluorescence quenching therefore increases the risk of false-positive results, decreasing the specificity of the technique. Interestingly, a recent publication showed that precipitation of hemoglobin with trichloroacetic acid prior to the measurement of 4-methylumbelliferone significantly increased the height of the output signal (Oemardien et al., 2011).

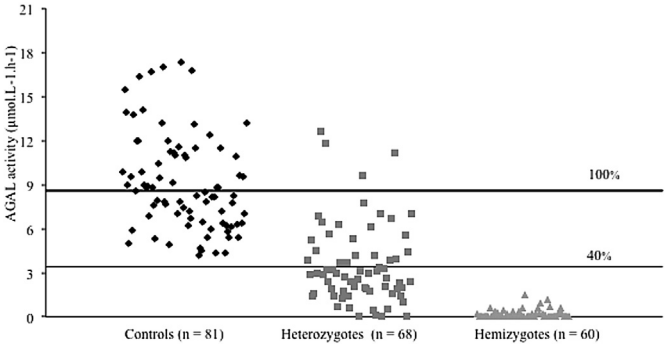

Fig. 1. Alpha-galactosidase (AGAL) enzyme activities in dried blood spots from controls and patients with Fabry disease.