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Determination of the lysosomal hydrolase activity in blood collected on filter paper, an alternative to screen high risk populations

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ABSTRACT

This study aimed to determine the enzymatic activity in dried blood samples collected on filter paper (DBS) for the diagnosis of the following diseases: Fabry, Pompe, Mucopolysaccharidosis type I (MPS I) and Mucopolysaccharosis type VI (MPS VI). DBS was used for high risk patientscreening, according to clinical suspicion. Plasma, leukocytes and cultured fibroblasts were used to confirm the diagnosis when necessary. Among the 529 DBS samples sent to the laboratory, 164 had abnormal results. Confirmatory materials of 73 individuals were rerouted. The frequency of diagnosis for lysosomal storage disorders was 5.9%. DBS is an alternative screening technique used in high risk populations, which should lead to earlier diagnosis for lysosomal storage disorders (LSDs), help patients get treatment sooner and improve the outcome of the disease.

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1. Introduction

Lysosomal storage disorders (LSDs) are a large group of more than 50 different inherited metabolic diseases that result from the defective function of specific lysosomal enzyme or defects in non-enzymatic lysosomal or non-lysosomal proteins involved in lysosome biogenesis. The progressive lysosomal accumulation of undegraded metabolites results in generalized cell and tissue dysfunction, and therefore, in multisystemic pathology (Mueller and Young, 2001). Most LSDs are autosomal recessive, except for Fabry disease, Danon disease and Hunter Syndrome, which are X-linked (Wilcox, 2004). These diseases are rare, with an estimated combined incidence of 1 in 7000 live births (Fletcher, 2006). It is believed that a large-scale screening may provide values closer to reality.

The biochemical diagnosis of LSDs is performed in different biological samples such as plasma, leukocytes or cultured fibroblasts (Parkinson et al., 2006). Nowadays, a new method is being used. It consists of the enzymatic analysis of dried blood spots on filter paper (DBS). This procedure has some advantages over other methods as it requires only a few drops of blood, facilitates packing and shipping, and presents less risk of biological contamination by laboratory personnel (Li and Tse, 2010). Many studies have been performed to ensure that this material is adequate for screening and/or diagnosis of LSDs.

There are different methods for determining the activity of lysosomal hydrolases in DBS (Carpenter and Wiley, 2002; Fuller et al., 2004; Umapathysivam et al., 2001) and it is important to consider more than one type of material for testing or diagnosis, when research is being conducted in patients with clinical suspicion of LSD (Staretz-Chacam et al., 2009). In general, fluorometry and liquid chromatography combined with tandem mass spectrometry (LC–MS/MS) techniques are widely used (Chamoles et al., 2001; Civalero et al., 2006; De Jesus et al., 2009; Gasparotto et al., 2009; Olivova et al., 2009).

The present study reports the experience of our laboratory in screening and diagnosis of Fabry and Pompe diseases and Mucopolysaccharidosis type I (MPS I) and type VI (MPS VI) in a period of two years.

2. Materials and methods

2.1. Samples

A total of 529 blood samples were collected on filter paper from April 2010 to May 2012. These samples were taken from patients (51% males, 49% females) with presented signs or symptoms of four different lysosomal storage disorders (Fabry disease, Pompe disease, Mucopolysaccharidosis I (MPS I) and Mucopolysaccharosis VI (MPS VI)). These signs and symptoms are described in Table 1. DBS and plasma, leukocytes or cultured fibroblasts, when necessary, were used to determine the enzymatic activity of alpha-galactosidase A (GAL, EC 3.2.1.22), alpha-glucosidase (GAA, EC 3.2.1.20), Arylsulphatase B (ASB, EC 3.2.6.12) and alpha-iduronidase (IDUA, EC 3.2.1.76).

Abbreviations: DBS, Dried blood spots collected on filter paper; MPS I, Mucopolysaccharidosis type I; MPS VI, Mucopolysaccharosis type VI; LSDs, Lysosomal storage disorders; LC/MS/MS, Liquid chromatography combined with tandem mass spectrometry; GAL, Alpha-galactosidase A; GAA, Alpha-glucosidase; ASB, Arylsulphatase B; IDUA, Alpha-iduronidase; ROC, Receiver Operating Characteristics.

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Table 1
Clinical signs and symptoms reported for some patients, n = 125.

Clinical findings	%
Angiokeratoma	31
Pain in extremities	30
Cardiac failure	23
Hypotonic	20
Hepatosplenomegaly	10
Gastrointestinal changes	6
Neurological changes	6
Skeletal changes	5
Renal failure	4
Cerebrovascular accident	4
Intolerance to heat/cold	4
Ocular symptoms	2
Liver changes	2
Respiratory changes	2
Bone pain	2
Hematologic changes	2
Other	1

All samples were sent by pediatricians, neurologists and geneticists from several regions of Brazil to the Laboratory of Lysosomal Storage Disorders at the Biochemistry Department of the Universidade Federal do Rio Grande do Sul (UFRGS) in Southern Brazil.

This study was approved by the Research Ethics Committee of the Universidade Federal do Rio Grande do Sul in accordance with the World Medical Association Declaration of Helsinki-Ethical Principles for Medical Research Involving Human Subjects.

2.2. Methodology

DBS (Whatman 903, Whatman, Kent, UK, www.whatman.com) samples were sent to the laboratory by mail. They were dried at room temperature and stored in self-sealing plastic bags containing desiccant to prevent dehydration.

Alpha-iduronidase was measured according to the method of Civallero et al. (2006) with a four time reduction volume. The analytical assays on DBS for beta-galactosidase and alpha-galactosidase were based on the protocols proposed by Castilhos et al. (2011) and de Castilhos et al. (2011). The alpha-glucosidase activity was measured based on Li et al. (2004), using 8 mM acarbose, the maltase-glucoamylase inhibitor. The assay solution comprised 0.2 M citrate-phosphate buffer (pH 4.0) with 0.6 g/L Triton X-100 and 10 mM substrate 4-methylumbelliferyl-α-D-glucoside. The analysis was reduced to 2.5 times.

In our experiments, all the chemicals and reagents were of analytical grade. All analyzes were performed in duplicate using a 1.2 mm filter paper punch (Harris Unicore-1.2 mm, Sigma, St. Louis, MO, USA) containing approximately 0.9 μL of whole blood. For incubation, fluorometer 96-well plates (Perkin-Elmer OptiPlate-96F) were used.

After incubation at 37 °C (Marconi MA-127 incubator), the reactions were stopped by the addition of alkaline buffer. A Spectramax M5 (Multi-Mode Microplate Reader Molecular Devices-M5) was used as a fluorescence reader (excitation 365 nm; emission 450 nm).

When a low level of DBS enzymatic activity was detected, new samples of peripheral blood were requested for alpha-iduronidase, alpha-galactosidase and ASB (10 mL heparinized whole blood) or skin biopsy for alpha-glucosidase (approximately 3 mm of forearm skin biopsy). Two milliliters of blood samples were centrifuged at 2000 rpm to obtain plasma and 8 mL to obtain leukocytes according to Skoog and Beck (1956). The samples were stored at −20 °C until the enzymatic analyzes were complete. To obtain cultured fibroblasts, the cells were prepared according to Coelho and Giugliani (2000) in Ham's F-10 medium supplemented with 10% fetal bovine serum. This procedure was used when the activity of alpha-glucosidase in DBS was below the range reference.

In the samples of leukocytes and fibroblasts, protein quantities were determined according to Lowry et al. (1951). Beta-galactosidase activity was measured (de Castilhos et al., 2011) to determine the feasibility of the material.

The alpha-glucosidase activity in cultured fibroblasts was measured as described by Hermans et al. (1991). This reaction occurred in acetic buffered medium (0.2 M pH 4.0) and in 2 mM 4-methylumbelliferyl-beta-D-glucopyranoside substrate (Sigma) after 1 hour incubation at 37 °C. The alpha-galactosidase activity was measured in plasma according to Morgan et al (1990) with 2 hour incubation at 30 °C with 0.5 M sodium acetate buffer, pH 4.8. The ASB activity was measured in leukocyte according to Kresse et al. (1982). The samples were incubated for 1 h and 30 min at 37 °C with 4-Nitrocatechol sulfate substrate (Sigma) in 0.5 M acetate buffer, pH 6.0. Enzymatic activity of alpha-iduronidase in plasma was determined by adding 2 mM 4-methylumbelliferyl-alpha-iduronide cyclohexylammonium salt (Glycosynth) in 50 mM sodium formate buffer, pH 2.8, according to Hopwood et al. (1979).

All reactions were incubated in a thermostat incubator (Marconi MA-127) and stopped by the addition of alkaline buffer. The reactions were performed in duplicate and a blank, with all reagents except the substrate, was used. Fluorescence was measured using 96-well plates at frequencies of 365 nm (excitation) and 450 nm (emission). The readings were corrected by blank subtraction and the activity was calculated using a standard curve of methylumbelliferone. Enzyme activities were expressed as nmol hydrolyzed substrate per hour (or 20 h-alpha-glucosidase) per milliliter of blood (nmol/h·mL^{-1}) to DBS and plasma. For leukocytes and fibroblasts the enzyme activities were expressed as nmol hydrolyzed substrate per hour per milligrams of protein $\text{nmol/h·mg protein}^{-1}$.

For all techniques used in DBS, plasma, leukocytes or fibroblasts, 20 samples from normal control (blood donors or volunteers), 52% male and 48% female with age ranging between 2 and 55 years old, were used to determine the reference range (maximum and minimum) and cutoff point with 100% sensitivity and specificity (ROC curve). Statistically significant differences ($p < 0.01$) were observed only in the alpha-galactosidase activities (DBS and plasma) between sexes. These differences were already observed in a previous work (Daitx et al., 2012). No statistically significant differences were observed in all enzymatic activities between the pediatric and adult groups.

3. Results

A total of 529 DBS samples from patients suspected of LSDs were screened and the results obtained as well as the protocol established for this study are presented in Fig. 1. After screening, 365 DBS samples

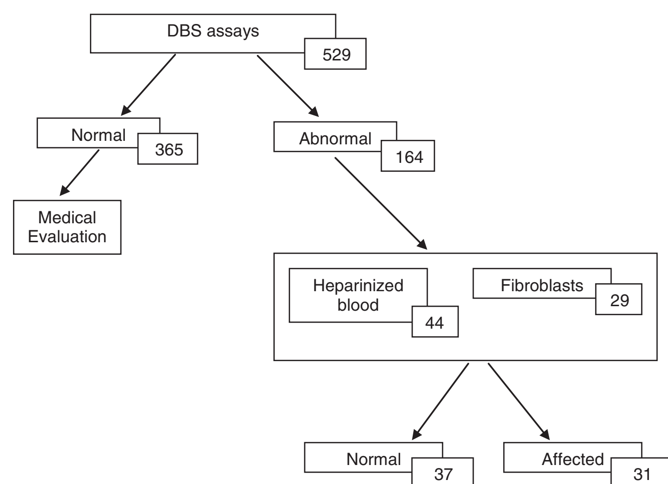

Fig. 1. Flowchart of results obtained in DBS and confirmatory samples sent to Laboratory of Lysosomal Storage Diseases.

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