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Beta-glucuronidase activity in dried blood spots: Reduced technique with biochemical parameters determined

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ABSTRACT

Introduction: Mucopolysaccharidoses (MPS) occur due to deficiency in the activity of enzymes that catalyze the breakdown of glycosaminoglycans. MPS VII is caused by deficiency of the beta-glucuronidase enzyme (GUSB).
Objectives: This study aimed to enhance the technique to measure GUSB activity by reducing the amount of reagents and the size of the DBS, as well as to determine some biochemical parameters of enzyme of healthy individuals.
Methods: The measurement of GUSB in 3 and 1.2 mm DBS (with reagents reduced 2.5- and fourfold) was correlated and the precision of the technique was tested. Optimal pH, Km and Vmax, and thermostability parameters were determined and time and temperature of sample storage were established.
Results: The correlations among the techniques were significant. Although the correlation coefficient was similar, fourfold reduction was selected. pH 4.4 had the highest enzyme activity. GUSB's Km was 1.25 mM, while Vmax was 594.48 nmol/h/mL. After pre-incubation of the sample at 60 °C, its activity dropped from 100% to 15.8% at 120 min. GUSB activity significantly decreased after 45 days of storage at 4, 25, and 37 °C.
Conclusions: This research allowed a previously described technique for MPS VII diagnosis to be adapted for smaller amounts of sample and reagents. That will facilitate the use of smaller amounts of samples, which may be used for other techniques and to save material. Given the importance of early MPS VII diagnosis due to the severity of the disease, using reliable diagnostic techniques in DBS is essential.

1. Introduction

Mucopolysaccharidoses (MPS) are inborn errors of metabolism, more specifically belonging to the group of lysosomal storage diseases (LSD), which occur due to deficiency in the activity of enzymes that catalyze the breakdown of glycosaminoglycans (GAGs) [1]. Mucopolysaccharidosis VII (MPS VII, OMIM #253220), called Sly syndrome, is caused by deficiency of the beta-glucuronidase enzyme (GUSB, EC 3.2.1.31) [1], which prevents the breakdown of glucuronic acid residues contained in the glycosaminoglycans dermatan sulfate, heparan sulfate, and chondroitin sulfate [2–5].

In most LSDs, the final diagnosis, determined by deficiency in enzyme activity measured in leukocytes samples [6], is performed during childhood and adulthood. However, persons affected by MPS VII may have hydrops fetalis at birth and survive for only a few months and only in rare cases do patients, with mild manifestations, survive [5,7].

The use of dried blood spots on filter paper (DBS) to measure enzyme activity in suspected LSD has been described [8–12] as advantageous compared to other samples such as leukocyte separation or fibroblast culture. Besides eliminating those steps of procedures, the methods in DBS allow a smaller volume of samples and reagents to be used, which enable several analyses to be performed simultaneously. Saving material for the investigation of other diseases and the ease of transportation and storage, allied to the fact the material for analysis can be collected right after birth, are additional upsides of the method [9]. Techniques with DBS are suitable for LSD screening, but positive diagnosis should be confirmed in plasma or leukocyte samples [13].

This study aimed to enhance the technique to measure GUSB enzyme activity by reducing the amount of reagents and the size of the DBS, as well as to determine the biochemical parameters of Km (Michaelis-Menten constant), Vmax (maximum velocity), thermostability, and sample storage time and temperature, therefore

Abbreviations: MPS, Mucopolysaccharidoses; GAGs, glycosaminoglycans; MPS VII, Mucopolysaccharidosis type VII; GUSB, beta-glucuronidase; Km, Michaelis-Menten constant; Vmax, maximum velocity; DBS, dried blood spots on filter paper; ANOVA, analysis of variance

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establishing a reference range for the enzyme's activity in samples of healthy individuals.

2. Materials and methods

2.1. Sample collection and storage

Sample size was estimated as at least eight samples to make up the analysis group using the statistical software MiniTab®16. The standard deviation of alpha-iduronidase enzyme activity in persons affected with mucopolysaccharidosis I was considered with margin of error of 5%. The sample n was described per experiment.

The blood samples employed were obtained from volunteer participants invited to take part in the research at the Blood Bank of the Clinics Hospital of Porto Alegre. Ten millilitres of blood were collected with heparin anticoagulant from each subject. The samples were homogenized and a disposable Pasteur pipette was used to place two drops of blood in each circle of a card made of Whatman 903® filter paper. This paper was dried at room temperature and stored in plastic bags containing desiccant at the temperature and times of each test.

2.2. Measuring GUSB enzyme activity

Enzyme activity measurement was adapted from the fluorimetric technique in 3 mm DBS described by Civallero et al. [8] using the artificial 4-methylumbelliferyl-beta-D-glucuronic acid substrate (Sigma). GUSB activity was expressed as nmol/h/mL.

2.3. Standardizing the technique to measure GUSB activity using 1.2 mm dried blood spots

Punch size was reduced from 3 mm to 1.2 mm and the reagents were reduced 2.5-fold and fourfold (Table 1). Pearson correlation tests were performed in 14 samples between the measure of GUSB activity in 3-mm DBS samples and the same activity in 1.2-mm DBS with reagents reduced 2.5-fold and in 1.2-mm DBS with reagents reduced fourfold. All fluorimetric analyses were performed in 96-well fluorimetric plates (OptiPlate-96 F, Perkin Elmer) and fluorescence was read in a Spectramax M5 (SpectraMax M5 Multi-Mode Microplate Readers - Molecular Devices).

2.4. Coefficients of variation to validate the GUSB enzyme activity measurement technique using lower amounts of reagents

After the methods were developed, the technique with the reagents reduced fourfold was selected and the technique's precision was verified by determining the inter-assay, interpersonal, and intra-assay coefficients of variation (CV) using ten DBS samples of healthy individuals.

Ten 1.2-mm DBS punches were used and enzyme activity was measured in the same enzymatic reaction, as a single test, to determine the intra-assay CV. In order to establish the inter-assay CV, enzymatic reactions were performed on five different days using the same DBS samples. The interpersonal CV analysis compared the results obtained in the analysis of the samples by two researchers simultaneously.

Table 1
Miniaturization of GUSB activity measurement method.

Reagents	3 mm	1.2 mm[a]	1.2 mm[b]
Water (μL)	50	20	12.5
DBS sample (mm)	3	1.2	1.2
4-Metilumbeliferil-β-D-glicuronic acid 10 mM (μL)	50	20	12.5
Incubation time (h) at 37 °C	2	4	4
Ethylenediamine 0.13 mol/L pH 11.3 (μL)	300	120	75

[a] Reduced 2.5 times.
[b] Reduced 4.0 times.

2.5. Determining GUSB's optimal pH

In order to determine GUSB's optimal pH, a 0.1 M sodium acetate buffer was prepared, which was used to solubilize the enzyme substrate at pH 2.0, 3.0, 4.0, and 5.0. After this first analysis, pH values were limited to 4.0, 4.2, 4.4, 4.6, 4.8, 5.0, 5.2, and 5.4.

2.6. Determining GUBS's Km and Vmax

To determine the Michaelis-Menten curve (substrate curve), solutions of the 4-methylumbelliferyl-beta-D-glucuronic acid substrate were prepared at 1.25, 2.50, 5.0, 10.0, and 12.5 mM through dilution in pH 4.4 0.1 M sodium acetate buffer. After the substrate curve was obtained, linearity was observed and new substrate concentrations points were established (0.25, 0.50, 0.75, 1.00, 1.25, and 1.50) to determine the reaction's Km and Vmax. Those parameters were calculated using the Lineweaver-Burk plot.

2.7. Studying the effect of temperature (thermostability) on GUSB activity

In order to study the effect of temperature on GUSB activity, first an inactivation temperature curve was built for the enzyme. The 1.2-mm blood-impregnated paper filter samples of healthy individuals underwent pre-incubation in a water bath (Marconi-MA 127) for 30 min at 40 °C, 50 °C, 60 °C, and 70 °C. After this period, enzyme activity was measured. The activity of the samples incubated at the aforementioned temperatures was compared with activity considered 100% (4 °C in pre-incubation for 30 min).

After the temperature curve was built, the temperature of 60 °C was chosen, whose samples best responded to inactivation, to proceed with the GUSB thermostability assays. For the GUSB thermostability assay, the samples were pre-incubated for 5, 10, 15, 30, 45, 60, 90, and 120 min at 60 °C prior to the assay to measure enzyme activity. The result of those samples incubated at 4 °C was considered 100% enzyme activity, i.e., total activity with no enzyme inactivation.

2.8. Establishing GUSB's reference range

GUSB's enzyme activity reference range was established based on 50 DBS samples of healthy individuals. The analyses were carried out using the miniaturized technique with the volume of reagents reduced fourfold.

2.9. Testing storage time and temperature

The storage time and temperature experiments were carried out by storing blood-impregnated paper filter samples from 12 individuals at − 20 °C, 4 °C, 25 °C, and 37 °C and measuring the enzyme activity at 0, 7, 14, 30, 45, and 60 days of storage.

2.10. Statistical analysis

The data were expressed as means ± standard deviation. In order to assess the association among the techniques, Pearson correlation coefficient was employed. The selected technique was validated by calculating the intra-assay, inter-assay, and interpersonal coefficients of variation, while the kinetic parameters Km and Vmax were calculated using Lineweaver-Burk plot.

The thermostability results obtained from the samples were compared using one-way ANOVA followed by Bonferroni test and the storage time and temperature analyses were compared using two-way ANOVA at $p < 0.05$. All analyses were performed using the software GraphPad Prism version 5.03.