Hurler-like Phenotype: Enzymatic Diagnosis in Dried Blood Spots on Filter Paper

Néstor A. Chamoles,* Mariana B. Blanco, Daniela Gaggioli, and Carina Casentini

Background: Clinical differentiation among mucopolysaccharidosis, oligosaccharidosis, and mucolipidosis II and III is difficult. We describe methods for the assay of 8 lysosomal enzymes in dried blood spots on filter paper that allow screening for 12 lysosomal storage diseases that present with a Hurler-like phenotype.

Methods: To test tubes containing 3-mm blood spots, we added elution liquid and fluorescent or radioactive substrate solution. After incubation at 37 ºC, the reaction was terminated by the addition of a stop buffer. The amount of hydrolyzed product was compared with a calibrator to allow the quantification of enzyme activity. Sample stability was studied during storage for 21 days and during shipment of samples. We measured enzyme activities in 85 healthy controls (35 newborn, 50 adult), 57 patients suffering from 11 lysosomal storage diseases, and 46 obligate carriers.

Results: Intra- and interassay CVs were <9% and <15%, respectively. Mean activity losses during transportation or storage for up to 21 days at 4 ºC were ≤27%. Enzyme activities in all patients were outside the ranges of values seen for carriers and controls.

Conclusions: The described methodology distinguishes between patients and controls with samples that are sufficiently stable to be mailed to the testing laboratory.

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Mucopolysaccharidosis (MPS),1 oligosaccharidosis, and mucolipidosis (MLP) II and III are expressed as progressive storage diseases that share many clinical features, such as coarse face, hepatosplenomegaly, bone dysplasia, and claw-hand deformities. These patients present with a peculiar appearance or Hurler-like phenotype. All of the disorders are associated with a wide spectrum of clinical phenotypes, ranging from mild to severe, and an impressive degree of overlap in clinical presentation. Thus, clinical differentiation among these disorders is difficult (1).

We describe methods for the assay of eight lysosomal enzymes in dried blood spots on filter paper (DBFP), that allow the screening of 12 lysosomal storage diseases manifested with a Hurler-like phenotype. The enzymes included in this protocol, the disorders produced by their dysfunction, the number of patients and carriers examined, and the references for the assay methods that have been adapted to DBFP are shown in Table 1. MPS III, or Sanfilippo syndrome type A, B, C, and D, and MPS IV A, or Morquio syndrome type A, can present with Hurler-like symptoms, particularly in the early-onset variants. The leukocyte diagnostic methods for these disorders could not be adapted to DBFP samples.

Materials and Methods

Samples

After informed consent of the patients or families, we obtained DBFP samples (Schleicher and Schuell No. 903) from 57 patients and 46 obligate carriers (Table 1). DBFP samples from 50 healthy adults (18–54 years) and 35 newborns (3–7 days postpartum) were used as controls. DBFP samples were stored at 4 ºC in plastic bags until analysis. The assays were performed no more than 72 h after blood sampling.

Chemicals

4-Methylumbelliferone, 4-methyl-umbelliferyl (4MU)-α-L-iduronide, d-saccharic acid-1, 4-lactone, 4MU-sulfate, 4MU-β-D-glucuronic acid, 4MU-β-D-galactoside, 4MU-α-L-fucoside, 4MU-α-D-mannoside, 4MU-β-D-mannoside, and 4MU-2-acetamido-2-deoxy-β-D-glucosaminide were obtained from Sigma Co. Tritiated iduronosyl sulfate anhydro-mannitol sulfate and Cellex E (anion-exchange resin) were supplied by Toronto Research Chemicals.

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1 Nonstandard abbreviations: MPS, mucopolysaccharidosis; MLP, mucolipidosis; DBFP, dried blood spots on filter paper; and 4MU, 4 methylumbelliferyl.
sented as elution liquid, substrate, and the incubation time, represented a mixture of the paper punch in the elution liquid and the substrate, which had been incubated separately. The tubes were incubated at 37 °C for different times in a slowly oscillating shaking water bath. The tubes were then placed in ice, and 300 μL of glycine-carbonate buffer (0.085 mol/L, pH 10.5) was added to stop the reaction. The filter paper did not need to be removed during the analysis. One blank tube was assayed for each sample. Blanks were prepared by adding 300 μL of stop buffer to a mixture of the paper punch in the elution liquid and the substrate, which had been incubated separately. The elution liquid, substrate, and the incubation time, represented as “EL”, “S”, and “IT”, respectively, for each enzyme assay were as follows.

(a) α-L-Iduroaminidase: IT, 20 h; EL, 40 μL of formate buffer (0.05 mol/L, pH 2.8), containing 0.3 μg of d-saccharic acid 1,4-lactone; S, 20 μL of 2 mmol/L 4MU-α-L-idoaminidase in distilled water.

(b) Arylsulfatase B: IT, 20 h; EL, 30 μL of distilled water and 20 μL of 15 mmol/L lead acetate in sodium acetate buffer (0.05 mol/L, pH 5.0); S, 50 μL of 10 mmol/L 4MU-sulfate in sodium acetate buffer (0.05 mol/L, pH 5.0).

(c) β-D-Glucuronidase: IT, 4 h; EL, 50 μL of distilled water; S, 50 μL of 10 mmol/L 4MU-β-D-glucuronic acid in sodium acetate buffer (0.1 mol/L, pH 4.8).

(d) β-D-Galactosidase: IT, 3 h; EL, 40 μL of citrate-phosphate buffer (0.05 mol/L, pH 4.4) in 45 g/L NaCl; S, 20 μL of 0.8 mmol/L 4MU-β-D-galactoside in distilled water.

(e) α-L-Fucosidase: IT, 20 h; EL, 30 μL of sodium citrate buffer (0.17 mol/L, pH 4.5); S, 50 μL of 1 mmol/L 4MU-α-L-fucoside in distilled water.

(f) β-Hexosaminidase: IT, 2 h; EL, 50 μL of citrate-phosphate buffer (0.022 mol/L, pH 4.4); S, 100 μL of 3 mmol/L 4MU-2-acetamido-2-deoxy-β-D-glucosaminide in citrate-phosphate buffer (0.022 mol/L, pH 4.4).

(g) α-D-Mannosidase: IT, 2 h; EL, 30 μL of sodium citrate buffer (0.17 mol/L, pH 4.4) containing 17.6 μg of zinc acetate; S, 50 μL of 10 mmol/L 4MU-α-D-mannoside in distilled water.

Fluorescence (excitation, 365 nm; emission, 450 nm) of the enzyme product 4MU was measured with a Farrand fluorometer model RF-2 (Farrand Optical). The fluorescence readings were corrected for blanks, and the results were compared with the fluorescence from a 4-methylumbelliferone calibrator. Enzymatic activities were expressed as micromoles of substrate hydrolyzed per liter of blood per hour.

**Fluorescence Enzyme Assays in DBFP Samples**

The detailed assay of α-L-idoaminidase in DBFP samples has been published elsewhere (2). Briefly, to duplicate 2-mL disposable test tubes containing a 3-mm blood spot (~3.6 μL of blood) obtained with a standard paper punch, we added elution liquid and substrate. After gentle mixing, the tubes were incubated at 37 °C for different times in a slowly oscillating shaking water bath. The tubes were then placed in ice, and 300 μL of glycine-carbonate buffer (0.085 mol/L, pH 10.5) was added to stop the reaction. The filter paper did not need to be removed during the analysis. One blank tube was assayed for each sample. Blanks were prepared by adding 300 μL of stop buffer to a mixture of the paper punch in the elution liquid and the substrate, which had been incubated separately. The elution liquid, substrate, and the incubation time, represented as “EL”, “S”, and “IT”, respectively, for each enzyme assay were as follows.

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(b) Arylsulfatase B: IT, 20 h; EL, 30 μL of distilled water and 20 μL of 15 mmol/L lead acetate in sodium acetate buffer (0.05 mol/L, pH 5.0); S, 50 μL of 10 mmol/L 4MU-sulfate in sodium acetate buffer (0.05 mol/L, pH 5.0).

(c) β-D-Glucuronidase: IT, 4 h; EL, 50 μL of distilled water; S, 50 μL of 10 mmol/L 4MU-β-D-glucuronic acid in sodium acetate buffer (0.1 mol/L, pH 4.8).

(d) β-D-Galactosidase: IT, 3 h; EL, 40 μL of citrate-phosphate buffer (0.05 mol/L, pH 4.4) in 45 g/L NaCl; S, 20 μL of 0.8 mmol/L 4MU-β-D-galactoside in distilled water.

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(f) β-Hexosaminidase: IT, 2 h; EL, 50 μL of citrate-phosphate buffer (0.022 mol/L, pH 4.4); S, 100 μL of 3 mmol/L 4MU-2-acetamido-2-deoxy-β-D-glucosaminide in citrate-phosphate buffer (0.022 mol/L, pH 4.4).

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**Iduronate Sulfatase Assay in DBFP Samples**

To duplicate 2-mL test tubes containing 3-mm blood spots, we added 50 μL of 16 mmol/L lead acetate as the elution liquid. After gentle mixing for 10 min at room temperature, we added 30 μL of tritiated iduronosyl sulfate anhydromannitol sulfate, 10 μCi/34.3 nmol, specific activity 291 mCi/mmol (Toronto Research Chemicals) (3), previously dissolved in 6.8 mL of sodium acetate buffer (0.33 mol/L, pH 4.5). The tubes were incubated for 20 h at 37 °C in a shaking-water bath. The reaction was stopped with 1 mL of 1 mmol/L sodium dibasic phosphate. The reaction mixture was applied to small columns, each containing 0.6 mL of Cellex E (anion-exchange resin) in water. The columns were washed with 2 mL of 1 mmol/L sodium dibasic phosphate and eluted with 5 mL of 70 mmol/L freshly prepared sodium formate. The eluted liquid was transferred to a scintillation vial, and 15 mL of optifluor (Packard Instrument Co) was added. The vials were counted for 5 min in a Tri-Carb Liquid Scintillation Analyzer (Model 1900 TR; Packard Instrument Co). One enzyme-free blank was assayed in duplicate for each assay. The disintegrations per minute (dpm) readings were corrected for blanks. Iduronate sulfatase activity

**Table 1. Lysosomal enzymes measured in DBFP samples.**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Disease</th>
<th>P/C</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-L-Iduroaminidase (EC 3.2.1.76)</td>
<td>MPS I</td>
<td>13/7</td>
<td>2</td>
</tr>
<tr>
<td>Iduronate sulfatase (EC 3.1.6.12)</td>
<td>MPS II</td>
<td>10/8</td>
<td>3</td>
</tr>
<tr>
<td>Arylsulfatase B (EC 3.1.6.1)</td>
<td>MSD</td>
<td>1/2</td>
<td>4</td>
</tr>
<tr>
<td>β-Glucuronidase (EC 3.2.1.31)</td>
<td>MPS VI</td>
<td>10/8</td>
<td>4</td>
</tr>
<tr>
<td>β-Galactosidase (EC 3.2.1.23)</td>
<td>MSD</td>
<td>1/2</td>
<td>4</td>
</tr>
<tr>
<td>α-Mannosidase (EC 3.2.1.24)</td>
<td>α-Mannosidosis</td>
<td>2/2</td>
<td>5</td>
</tr>
<tr>
<td>α-L-Fucosidase (EC 3.2.1.51)</td>
<td>Fucosidosis</td>
<td>NA</td>
<td>5</td>
</tr>
<tr>
<td>β-Hexosaminidase (EC 3.2.1.30)</td>
<td>Sandhoff</td>
<td>3/3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Mucolipidosis II/III</td>
<td>5/4</td>
<td></td>
</tr>
</tbody>
</table>

*P, patients; C, carriers; NA, not available.*
was expressed as nanomoles of substrate hydrolyzed per liter of blood per hour.

**Results**

The effect of variable incubation times on the enzymatic activities is shown in Fig. 1. The extended period of incubation provided enough enzyme products to ensure reliable fluorometer readings. Initially, assays were always performed with one and two punched 3-mm blood spots. The determinations were also made at two different incubation times. Because we did not obtain any significant variation, these steps were later omitted. The possibility of an inhibitor of the enzymatic reactions in the filter paper matrix was eliminated by mixing experiments for each one of the enzymes studied (data not shown).

The enzyme activities of healthy adult and newborn controls, obligate carriers, and patients are reported in Tables 2 and 3. The newborn enzyme activities were higher than those of control adults for six enzymes (Table 2). There was no overlap among the results of patients and carriers or controls. The GM1 gangliosidosis and Sandhoff carriers studied in this experiment showed intermediate degrees of enzyme activity. However, there was a variable overlap between the heterozygotes and the control group for the other reported enzymes. The residual enzyme activities found in DBFP samples from homozygotes are shown in Table 3, expressed as a percentage of the mean of adult controls.

The intraassay CVs from healthy controls (n = 10) were ≤6% and ≤9% for the fluorescent and radioactive methods, respectively. The interassay CVS for DBFP samples studied on five different occasions within 1 month were ≤9% for the fluorescent methods and ≤14% for the iduronate sulfatase assay.

A positive correlation was found between the leukocyte count in blood and the degree of enzyme activity measured in DBFP samples. The decreases in enzyme values related to a low leukocyte count can be easily identified by simultaneous assay of the activities of several enzymes with preservation of their ratios. Activities of arylsulfatase B and \( \beta \)-galactosidase of 18.5 and 42.1 \( \mu \text{mol/L of blood per hour} \) (aryl sulfatase B/\( \beta \)-galactosidase ratio, 0.4), respectively, with a leukocyte count of 10 100/mm\(^3\), changed to 9.2 and 22.2 \( \mu \text{mol/L of blood per hour} \) (aryl sulfatase B/\( \beta \)-galactosidase ratio, 0.4), respectively, with a leukocyte count of 6000/mm\(^3\). Similar results have been observed in two samples stored incorrectly.

The different enzyme activities present in plasma and blood cells explain the nonlinear correlation between the enzyme activities from DBFP and leukocyte samples (data not shown).

There were no significant changes in enzyme activity of DBFP samples after storage for 21 days at \(-20\) °C, 4 °C, or 25 °C. To verify the stability of these enzymes under usual mailing conditions, DBFP samples from four controls, two GM1 gangliosidosis patients, two gangliosido-

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**Fig. 1.** Enzyme activities measured in DBFP samples as a function of incubation time.

Each symbol represents one assay performed on 3-mm filter paper punched from dried blood spots. (A), healthy newborn (line 1; \( \square \)),\( y = 0.7089x + 3.52 \) \( (R^2 = 0.9963) \); healthy adult (line 2; \( \bigcirc \)), \( y = 0.405x + 2.1 \) \( (R^2 = 0.9957) \); GM1 carrier (line 3; \( \triangle \)), \( y = 0.121x + 1.8 \) \( (R^2 = 0.9661) \). (B), healthy newborn (line 1; \( \square \)), \( y = 245.4x + 110.14 \) \( (R^2 = 0.9932) \); healthy adult (line 2; \( \bigcirc \)), \( y = 164.54x + 38.63 \) \( (R^2 = 0.9965) \); MPS II carrier (line 3; \( \triangle \)), \( y = 64.66x + 49.048 \) \( (R^2 = 0.9934) \). \( \bigstar \), activity in GM1 patient in A and MPS II patient in B.

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**Table 2.** Enzyme activities\(^a\) from healthy individuals.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Healthy newborns</th>
<th>Healthy adults</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Range</td>
</tr>
<tr>
<td>( \alpha )-Iduronidase</td>
<td>35</td>
<td>3.9–10.2</td>
</tr>
<tr>
<td>Iduronate sulfatase(^b)</td>
<td>12</td>
<td>205–475</td>
</tr>
<tr>
<td>Arylsulfatase B</td>
<td>35</td>
<td>9.0–40.2</td>
</tr>
<tr>
<td>( \beta )-Glucuronidase</td>
<td>35</td>
<td>131–426</td>
</tr>
<tr>
<td>( \beta )-Galactosidase</td>
<td>35</td>
<td>25.2–73.6</td>
</tr>
<tr>
<td>( \alpha )-Mannosidase</td>
<td>35</td>
<td>40–198</td>
</tr>
<tr>
<td>( \alpha )-Fucosidase</td>
<td>35</td>
<td>27–110</td>
</tr>
<tr>
<td>( \beta )-Hexosaminidase</td>
<td>35</td>
<td>553–1028</td>
</tr>
</tbody>
</table>

\(^a\) Except where noted otherwise, range and mean ± SD values are expressed as \( \mu \text{mol/L of blood per hour} \).

\(^b\) Expressed as \( \mu \text{mol/L of blood per hour} \).
sis carriers, two Maroteaux-Lamy patients, two Maroteaux-Lamy carriers, two Hunter patients, and two Hunter carriers were sent by air mail to the following locations: Sao Paulo, Brazil; Toronto, Canada; Brussels, Belgium; and Christchurch, New Zealand. The samples were sent back to our laboratory during our summer season (temperature, 28–37 °C). The turnaround times were 12, 15, 16, and 18 days, respectively. The mean enzyme activities from DBFP samples obtained from controls, carriers, and patients were reduced by almost the same percentage, which in no case was >27% of the initial activity (Fig. 2). The reduction did not modify the differentiation of patients from controls and carriers. β-Galactosidase, β-glucuronidase, and iduronate sulfatase were the most sensitive to the effect of shipment. Should DBFP samples from a patient under investigation be sent by mail to a specialized laboratory for analysis, it will be necessary to simultaneously send DBFP samples from a nonrelated control and an obligate carrier for comparison. This procedure will point out any unexpected decrease of the enzyme activities produced by transportation.

### Discussion

Our laboratory introduced the diagnosis of lysosomal storage diseases in DBFP samples (2, 4). DBFP analysis offers several advantages over whole blood samples, especially in terms of cost and ease of transportation and the suitability of the sample collection method for neonates where obtaining larger blood samples is not always convenient or possible (5). Additionally, DBFP methods could be applied to diagnose these disorders in potential patients from large areas of the world that lack specialized laboratories. The whole panel of enzymatic activities for the diagnosis of MPS, oligosaccharidosis, and MLP can be carried out with four or five DBFP samples in 48–72 h. Minimal activity losses occurred during 21 days of storage and during shipment of samples. These losses did not impair the differentiation of patients from controls. Twenty-one days is usually enough time to mail the DBFP sample to specialized laboratories for analysis.

Treatment of some of the genetic disorders expressing a Hurler-like phenotype became possible by bone marrow transplantation (6), enzyme replacement therapy (7), and gene modification (8). The effectiveness of these therapies, particularly for MPS involving the central nervous system, may rely heavily on early diagnosis of the disorders. Early diagnosis of the patient will allow clinicians to take advantage of the period of natural suppression of the immune system of the neonate to maximize the chances for a successful bone marrow engraftment (9). The convenience of treating presymptomatic patients and, at present, the possibility of an early diagnosis may make it reasonable to discuss the use of this methodology for a pilot newborn-screening program. For such a program, microplate adaptation and method automation could be easily performed.
In conclusion, the presented methodology is reliable and sensitive for measuring lysosomal enzyme activities in DBFP samples. DBFP methods can simultaneously measure 50 or more samples in duplicate in each assay, with no need for homogenization or protein assay procedures as is required for isolated leukocytes. All reagents are commercially available, no special or costly equipment is needed, and a quality-control program for the enzyme assays could be easily organized. Our preliminary results suggest that these methods can be applied to screen Hurler-like patients for lysosomal enzymes deficiencies. In abnormal cases, a blood sample for leukocyte isolation or a fibroblast skin culture should be requested for further characterization of the biochemical and molecular phenotypes of the disorder. To further validate this method, it will be important to study a larger population of patients and carriers. However, because of the low frequency of these disorders, such studies will be possible only with the cooperation of several specialized centers around the world.

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