Detection of Mucopolysaccharidosis Type II by Measurement of Iduronate-2-Sulfatase in Dried Blood Spots and Plasma Samples

Caroline J. Dean, Michelle R. Bockmann, John J. Hopwood, Doug A. Brooks, and Peter J. Meikle

Background: Mucopolysaccharidosis type II (MPS II) is a lysosomal storage disorder related to a deficiency in the enzyme iduronate-2-sulfatase (IDS). Clinical trials of enzyme replacement therapy are in progress, but effective treatment will require screening assays to enable early detection and diagnosis of MPS II. Our study evaluated the diagnostic accuracy of IDS protein and enzyme activity measurements in dried blood spots and plasma.

Methods: We collected dried-blood-spot and plasma samples from unaffected control individuals and from MPS II patients. We measured IDS protein concentration with a 2-step time-delayed dissociation-enhanced lanthanide fluorescence immunoassay. To measure enzyme activity, we immobilized anti-IDS antibody on microtiter plates to capture the enzyme and measured its activity with the fluorogenic substrate 4-methylumbelliferyl sulfate.

Results: Dried-blood-spot samples from MPS II patients showed an almost total absence of IDS activity (0–0.075 μmol·h⁻¹·L⁻¹) compared with control blood spots (0.5–4.7 μmol·h⁻¹·L⁻¹) and control plasma (0.17–8.1 μmol·h⁻¹·L⁻¹). A dried-blood-spot sample from only 1 of 12 MPS II patients had detectable concentrations of IDS protein (24.8 μg/L), but no IDS protein was detected in plasma from MPS II patients. Ranges for IDS protein in control samples were 25.8–153 μg/L for blood spots and 22.8–349.4 μg/L for plasma.

Conclusion: Measurement of the IDS protein concentration and enzyme activity (as measured by a simple fluorogenic assay with an immune capture technique) enables identification of the majority of MPS II patient samples from both dried blood spots and plasma samples.

Mucopolysaccharidosis II (MPS II; Hunter syndrome) is an X-linked, recessively inherited, lysosomal storage disorder (LSD) (1). MPS II is caused by accumulation of the glycosaminoglycans heparan sulfate and dermatan sulfate, which occurs as a result of a deficiency of the lysosomal enzyme iduronate-2-sulfatase (IDS; EC 3.1.6.13). Clinically, MPS II patients can present with variable symptoms, with disease categories ranging from severe to attenuated forms (1). Patients with a severe phenotype have pronounced neurologic impairment and often die in early childhood, whereas individuals with an attenuated phenotype may have little or no neurologic impairment and can survive into adulthood. Other symptoms include skeletal deformities, organomegaly, short stature, stiff joints, and coarse facial features (1). At least 309 IDS gene mutations have been cataloged in the Human Gene Mutation Database (http://www.hgmd.org) (2), and genetic heterogeneity is presumed to underlie most variations in clinical phenotype. However, genotype/phenotype studies have not provided the correlations required to accurately predict disease severity and progression in all MPS II patients (3–5).
Bone marrow transplantation is a treatment option for some MPS II patients, but it is not a panacea, as relatively few patients at the severe end of the clinical spectrum have positive outcomes (6). Enzyme replacement therapy has been successfully used in humans to treat several other LSDs, including Gaucher disease (7), Fabry disease (8), MPS I (9), and MPS VI (10). The correction of glycosaminoglycan storage by enzyme replacement therapy in a mouse model of MPS II (11) was followed by human clinical trials, which are ongoing. Because these treatments will probably be most successful if given early, before the onset of irreversible pathology, an effective method for the identification of MPS II patients is needed. Protein markers have already been effective for the detection of individuals with other LSDs (12–15). Assays of IDS activity (16–18) and a direct assay for dried blood spots (19) have been described, but a sensitive assay to quantify IDS protein from a 3-mm dried blood spot has not been reported. Because IDS does not efficiently hydrolyze the fluorescent substrate 4-methylumbelliferyl sulfate (4MU sulfate) under uncaptured assay conditions, existing assays rely on specialized or radiolabeled substrates (17), complex substrate/product detection protocols (19), or additional enzyme-linked systems (16, 18). An assay using the readily available 4MU sulfate would facilitate the early identification of MPS II patients. We describe an immunoassay for IDS protein and a solid-phase fluorometric assay that uses a polyclonal antibody to capture IDS and 4MU sulfate to detect enzyme activity, and we report on the diagnostic accuracy of these assays.

**Materials and Methods**

Recombinant human IDS was isolated from Chinese hamster ovary expression cells and purified by DEAE ion-exchange and phenyl-Sepharose chromatography to yield IDS, with characteristics similar to those described previously (20). Purified recombinant IDS was kept at 4 °C before use. A sheep anti-IDS polyclonal antibody was produced against this IDS and purified from serum by protein G chromatography and affinity chromatography with recombinant IDS protein, as described previously (5). The affinity-purified antibody was absorbed with sheep IgG coupled to a 1-mL Hitrap N-hydroxysuccinimide-activated HP affinity column (Amersham Pharmacia Biotech; 2 mg of sheep IgG on a 1-mL column), prepared according to the manufacturer’s instructions. Purified antibody was stored frozen at −20 °C before use. The sheep polyclonal was labeled with europium as described previously (21) and stored at 4 °C. Recombinant human N-acetylgalactosamine 4-sulfatase was expressed in Chinese hamster ovary cells (22), purified by monoclonal antibody affinity chromatography (13), and stored at 4 °C before use. Unless stated otherwise, all other reagents were of analytical grade and were obtained from Sigma Chemical Company.

**Patient Samples**

The Human Research Ethics Committee of the Children, Youth and Women’s Health Service, Adelaide, Australia, approved the use of dried-blood-spot and plasma samples in this study. Retrospective dried-blood-spot (3-mm diameter) and plasma samples from unaffected consecutive control individuals (age range, 0.5–50.5 years) and from MPS II–affected individuals (age range, 0.6–9.7 years), in whom MPS II was diagnosed enzymatically with a radiolabeled oligosaccharide substrate (23), were from specimens submitted to the National Referral Laboratory for the Diagnosis of Lysosomal, Peroxisomal and Related Genetic Disorders (Department of Genetic Medicine, Children, Youth and Women’s Health Service, Adelaide). Plasma samples were stored at −20 °C before use. Blood samples were also collected, with informed consent, from unaffected human controls (age range, 22.8–55.7 years) within the Department of Genetic Medicine. These latter samples were collected to evaluate several potential diagnostic assays for LSDs. Blood samples were collected into either EDTA or heparin. Dried-blood-spot samples were stored in sealed plastic bags inside plastic containers with desiccant at −20 °C up to 4 years before use. Clinical follow-up data were not available for the MPS II or control individuals, and the disease diagnoses of sample donors were known to persons executing IDS protein and enzyme activity assays.

**Measurement of IDS Protein by Immunoassay**

We measured IDS protein in dried blood spots and plasma samples with a 2-step time-delayed, dissociation-enhanced, lanthanide fluorescence immunoassay (DELFIA®). Microtiter plates (Immulon 4; DYNEX Technologies, Inc.) were coated with 100 μL of sheep anti-IDS polyclonal antibody at a concentration of 5 mg/L in 0.1 mol/L NaHCO₃ (pH 8.3) and incubated, without shaking, overnight at 4 °C. Plates were then washed 6 times (0.8 mL each time) with wash buffer [0.02 mol/L Tris-HCl, 0.15 mol/L NaCl, 20 mmol/L diethylene-triamine pentaacetic acid, 0.1 mL/L Tween 40 (BDH), and 0.02 g/L Thimerosal (pH 7.8)] in a DELFIA plate washer (1296-026; Perkin-Elmer Life and Analytical Sciences). Dried blood spots (duplicate samples) were placed in the coated microtiter wells with 100 μL of assay buffer [0.05 mol/L Tris-HCl, 0.15 mol/L NaCl, 20 μmol/L diethylene-triamine pentaacetic acid, 0.1 mL/L Tween 40, 5 g/L bovine serum albumin (BSA), 0.5 g/L bovine γ-globulin, and 0.5 g/L sodium azide (pH 7.8)]. Plasma samples (8 μL/well, assayed in duplicate) were diluted with assay buffer and added in a final volume of 100 μL/well. Liquid calibrators of purified IDS were included on every assay plate. The plates were covered and incubated at room temperature for 1 h with shaking (amplitude 5; Milenia Micromix 4 plate shaker; Model 602002; DPC), then placed at 4 °C overnight without shaking, before a 1-h incubation with shaking at room temperature. The blood spots were removed by aspiration, and the plates washed...
(6 times) with wash buffer. Europium-labeled anti-IDS polyclonal antibody was diluted in assay buffer (100 μL; final concentration, 200 μg/L) and added to each well. The plates were incubated overnight as described above. After washing (6 times) with wash buffer, DELFIA enhancement solution (200 μL/well; Wallac) was added to the plates and incubated at room temperature for 10 min with shaking. Fluorescence was measured on a DELFIA 1234 research fluorometer (Wallac). The IDS concentrations in the blood spots and plasma were calculated with spline fit curves generated by Multicalc Data Analysis software (Ver. 2.4; Wallac).

MEASUREMENT OF IDS ACTIVITY BY FLUOROGENIC IMMUNE-CAPTURE ASSAY

Microtiter plates were coated with affinity-purified sheep anti-IDS polyclonal antibody as described above, then washed (6 times) with 0.02 mol/L Tris-HCl (pH 7.0), containing 0.25 mol/L NaCl. Blood spots, plasma samples, and recombinant IDS calibrators were assayed in duplicate and diluted in 100 μL of phosphate-buffered saline (10 mmol/L Na2HPO4/NaOH, 138 mmol/L NaCl, 2.7 mmol/L KCl) containing 0.5 mL/L Tween 20, 5 g/L BSA, and 0.5 g/L γ-globulin. To demonstrate linearity, a 9-point calibration curve of recombinant human IDS (0–2000 pg/well) was run on every plate. Plates were covered and incubated at room temperature for 1 h with shaking, stored overnight at 4 °C without shaking, and then incubated again at room temperature for 1 h with shaking. Blood spots were removed by aspiration, and the plates were washed (twice) with 0.02 mol/L Tris-HCl and 0.25 mol/L NaCl (pH 7.0). Enzyme activity of the immune-captured IDS was determined by the addition of 5 mmol/L 4MU sulfate (stored at −20 °C) in 0.1 mol/L sodium acetate buffer (pH 5.6) containing 10 g/L BSA to each well. Plates were sealed and shaken for 1 min and then incubated without shaking for 24 h at 37 °C. Reactions were stopped by the addition of 100 μL of 0.2 mol/L glycine, 0.125 mol/L Na2CO3, and 0.16 mol/L NaOH (pH 10.7). Plates were shaken for an additional 10 min, and fluorescence was read on a Wallac Victor2 1420 Multilabel Counter (Perkin-Elmer Life and Analytical Sciences). With each analysis, a point calibration of 4-methylumbelliferone (710 pmol) was determined in duplicate. The enzyme activities of the samples were calculated by reference to the point calibration and corrected for incubation time (24 h) and sample volume (blood spots, 3 μL; plasma samples, 8 μL).

STATISTICAL ANALYSIS

Inter- and intraassay CVs were calculated for results from plasma and blood spots obtained from 2 different controls. Data were analyzed with SPSS (Ver. 11.0 for Windows) statistical software (SPSS Inc.).

Results

DEVELOPMENT OF IDS ASSAYS

The IDS protein and activity calibration curves were linear over the range 0–2000 pg/well (Fig. 1). We calculated the CV for the IDS protein assay by use of 10 replicates of each IDS calibrator (7.8 to 2000 pg/well), with 2-fold dilutions, and used a precision profile of CV against IDS concentration to determine the working range of the IDS protein assay (defined as protein concentrations with CVs <10%), which was 31.3 to 2000 pg/well. Samples with lower concentrations were deemed to have no detectable protein and assigned a zero concentration of IDS protein. The detection limit for IDS activity was defined by the concentration of activity required to give a signal 2 SD above the mean of the assay blank (n = 12). Any sample with activity below this value was deemed to have no detectable activity, and a zero degree of IDS activity was reported.
Intraassay CVs for the IDS protein assay, calculated from results for 2 different plasma samples with 20 replicates for each, were <6%. Intraassay CVs for the IDS protein assay, from results for 2 different blood-spot samples, were 9.6% and 9.2% (n = 20). Intraassay CVs for IDS activity, from 1 blood spot and 1 plasma sample, were 11% and 7.5%, respectively (n = 10). Intraassay CVs (n = 6) for the IDS protein were 13% and 10% for 2 plasma samples and 18% and 16% for 2 blood-spot samples. Interassay CVs (n = 6) for IDS activity were 9.6% for a plasma sample and 8.6% for a blood-spot sample.

We determined the stability of IDS in plasma (n = 61) and blood spots (n = 64) stored at −20 °C by assaying control samples stored for up to 4 years. No statistically significant correlations were seen between sample age and either IDS protein concentration or activity (data not shown). Only samples stored for <4 years were used in subsequent analyses for both the IDS protein concentration and activity assays. There was a significant correlation between IDS protein concentration and enzyme activity (Fig. 2 for both plasma [n = 38; Spearman correlation coefficient (r_s) = 0.732; P > 0.01, two-tailed] and blood-spot samples (n = 51; r_s = 0.751; P > 0.01, two-tailed).

The IDS enzyme did not hydrolyze the 4MU sulfate in an uncaptured activity assay (i.e., enzyme not captured on an antibody-coated microtiter well; data not shown). Similarly, the addition of various concentrations (1, 5, and 10 mg/L) of anti-IDS sheep polyclonal antibody to IDS protein in solution did not produce activity against 4MU sulfate (data not shown). There was negligible cross-reactivity with N-acetylgalactosamine 4-sulfatase in the immune-capture activity assay. N-Acetylgalactosamine 4-sulfatase (2000 pg/well) activity against the 4MU sulfate was only 3% that of IDS added at the same concentration (data not shown).

**Quantification of IDS Protein and Activity in Control and MPS II Plasmas and Blood Spots**

We investigated the relationship between the age of the control individuals and the concentration of IDS protein or enzyme activity was investigated. IDS protein concentrations decreased significantly with age for both plasma (n = 38; r_s = −0.578; P > 0.01, two-tailed) and blood spots (n = 51; r_s = −0.325; P > 0.05, two-tailed). We also found a significant decrease with increasing age for IDS activity in both plasma samples (n = 59; r_s = −0.453; P > 0.01, two-tailed) and blood spots (n = 62; r_s = −0.570; P > 0.01, two-tailed), but the lower limits did not change for either activity or protein concentrations over the range 0.5–55.7 years of age (data not shown).

In plasma, MPS II patient samples were distinguishable from control samples for both IDS protein concentration (Fig. 3A) and enzyme activity (Fig. 3B). One of the controls had an extremely high plasma protein concentration and activity (349.4 µg/L and 8.1 µmol·h⁻¹·L⁻¹, respectively); another control had low plasma IDS activity (0.168 µmol·h⁻¹·L⁻¹), but this value was still 2-fold higher than that of the MPS II patient with the highest residual enzyme activity.

The control with high plasma IDS protein concentration and enzyme activity also had high values in blood spots (Fig. 4); however, no clinical data were available for this individual. In blood spots, 11 of 12 MPS II patients had no detectable protein (Fig. 4A). One MPS II patient blood spot did have an IDS protein concentration of 24.8 µg/L, a value just below the lowest control value (25.8 µg/L). No genotype or clinical data were available for the patient who provided this sample, which along with 11 other MPS II patient samples had negligible IDS activity in blood spots and was clearly distinguishable from control samples (Fig. 4B).

**Discussion**

Enzyme replacement therapy for the treatment of MPS II patients is likely to be available in the near future. Early diagnosis and treatment of asymptomatic individuals will be imperative to maximize the efficacy of this therapy.
Current diagnostic techniques for MPS II involve the screening of urine to detect patterns of glycosaminoglycans and/or oligosaccharides, followed by a specific enzyme assay in either leukocyte or cultured skin fibroblast extracts. These methods are not amenable to high-throughput, large-scale screening programs. Thus, changing diagnostic requirements for this disease have prompted the development of the new assays reported here.

The method for quantification of IDS protein reported here was more sensitive than previously reported assays, with a working range of 31 to 2000 pg/well compared with a lower limit of detection of 1000 pg/well reported by Parkinson et al. (5) for nondenatured IDS and 5000 pg/well reported by Villani et al. (25). The current assay has the sensitivity to quantify IDS protein from a single 3-mm dried blood spot. Furthermore, only 8 μL of plasma was required in the current assays compared with 50 μL used previously (5). The large increases in assay sensitivity can be attributed to the use of the DELFIA detection technology instead of the less sensitive horseradish peroxidase detection methods (25) and to the use of a polyclonal antibody for both capture and detection steps.

A dried-blood-spot assay for IDS activity has been described previously (19), but this assay required the use of lead acetate to inhibit other lysosomal enzymes, a specific radiolabeled substrate, and an ion-exchange column step, making it impractical for large-scale screening applications. The novel fluorogenic immune-capture activity assay described here uses a commercially available 4MU sulfate substrate that could be measured directly in the reaction well, providing a simplified procedure amenable to high-throughput screening applications.
The immune assays for IDS protein and activity described here enabled the differentiation of all MPS II individuals from controls by use of either plasma or dried-blood-spot samples. No IDS protein was detected in any of the 9 MPS II plasma samples or in 11 of 12 MPS II blood-spot samples. One MPS II blood spot had detectable IDS protein, with a concentration just below the control range, although this sample had no IDS activity. This finding was consistent with previous reports of mutations such as K347T, 473delTCC, and N265I, which were shown to produce inactive IDS protein (26).

Of the sulfatases involved in LSDs, only arylsulfatase A (galactose-3-sulfatase) and arylsulfatase B (N-acetyl-galactosamine 4-sulfatase) efficiently hydrolyze 4MU sulfate. IDS, sulfamidase, N-acetylglucosamine-6-sulfatase, and galactose-6-sulfatase show little activity toward 4MU sulfate (27, 28). It appears that the capture of IDS by the antibody coated to the solid phase is critical for this enzyme to react with the 4MU sulfate substrate, because no reactivity was detected in the absence of capture antibody or when the antibody and enzyme were together in solution. Surface adsorption has been known to alter protein characteristics (29), and presumably, interaction between the protein and the adsorbed capture antibody led to an adsorption-induced conformation change, enhancing enzyme activity. Of note, a similar system for immune capture did not show increased activity with sulfamidase and 4MU sulfate (unpublished observations).

The methods for IDS protein and activity measurements described here are simple to perform, have potential as diagnostic assays, and are adaptable to large-scale, high-throughput protocols. We estimate that the cost for production and purification of 2.5 g of sheep anti-IDS polyclonal antibody, enough to cover 5 million newborns (annual US birth rate), to be approximately AUS $0.01 per sample. This cost includes chromatography columns for purification (approximately AUS $4000.00), 100 mg of IDS protein (approximately AUS $20 000.00), and labor (approximately AUS $26 000.00), to give a final cost of AUS $50 000.00. The preferred use of either enzyme activity or protein concentration determinations will depend to a large degree on the application. For a diagnostic assay, the use of enzyme activity measurements on dried blood spots or plasma would enable identification of all patients, including those with significant concentrations of inactive protein. In this study, 1 of the 19 MPS II individuals (5%) had IDS protein concentrations that were within the reference interval; therefore, MPS II would not be identified in this patient from a single IDS protein assay. We previously reported that the lysosomal storage of mucopolysaccharides leads to an increase in the number and size of lysosomal vacuoles (32). Furthermore, we reported alterations to other lysosomal protein concentrations such as LAMP-1 (21), LAMP-2 (33), and saposin C (34) in several LSDs, including MPS II. Multiplexing of the IDS protein assay with another 10 lysosomal proteins has been reported (35). Thus, we anticipate that alterations in multiple lysosomal proteins (a protein profile) will enable differentiation of patients with MPS II and other LSDs from the control population even if their mutant protein concentrations are within reference intervals. This technology could provide a platform for the further development of a newborn-screening program for LSDs.

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References


