

Tandem Mass Spectrometry for the Direct Assay of Lysosomal Enzymes in Dried Blood Spots: Application to Screening Newborns for Mucopolysaccharidosis I

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BACKGROUND: Treatments now available for mucopolysaccharidosis I require early detection for optimum therapy. Therefore, we have developed an assay appropriate for newborn screening of the activity of the relevant enzyme, α -L-iduronidase.

METHODS: We synthesized a new α -L-iduronidase substrate that can be used to assay the enzyme by use of tandem mass spectrometry together with an internal standard or by fluorometry. The assay uses a dried blood spot on a newborn screening card as the enzyme source. The assay protocol uses a simple liquid-liquid extraction step before mass spectrometry. We optimized enzyme reaction conditions and procedures for the assay, including the concentration of substrate, the reaction pH, the incubation time, and mass spectrometer operation. We also assessed inter- and intraassay imprecision.

RESULTS: When the assay was tested on dried blood spots, the α -L-iduronidase activity measured for 5 patients with mucopolysaccharidosis I was well below the interval found for 10 randomly chosen newborns. Inter- and intraassay imprecision were <10%. The synthesis of the α -L-iduronidase substrate is practical for use on a scale needed to support newborn screening demands.

CONCLUSIONS: This newly developed tandem mass spectrometry assay has the potential to be adopted for newborn screening of mucopolysaccharidosis I. This assay has advantages over a previously reported assay also developed in this laboratory and has the potential to be performed in a multiplex fashion to measure sev-

eral lysosomal enzymes relevant to treatable lysosomal storage diseases.

Mucopolysaccharidosis type I (MPS-I)⁴ is a lysosomal storage disorder caused by the deficiency of α -L-iduronidase (IdA; EC 3.2.1.76) activity. MPS-I can manifest 3 major clinical phenotypes, Hurler, Scheie, and Hurler-Scheie syndromes. IdA is essential for the degradation within lysosomes of the glycosaminoglycans dermatan and heparan sulfate. Because symptoms of MPS-I may not be recognized early in life, the diagnosis of MPS-I is a challenging task. Enzyme replacement therapy and bone marrow transplantation are treatments that have been developed for this disease, and both are beneficial if performed early (1, 2). Because early detection is necessary for optimum clinical response to therapy, there is a need for screening tests for the early recognition of MPS-I. Fluorometric, radiometric, and electrospray ionization-tandem mass spectrometry (ESI-MS/MS) assays have been developed (3, 4). The latter offers the capability of assaying the products of several enzymes by single infusion into the mass spectrometer (multiplexing).

MS/MS is used in newborn screening programs to quantify the concentrations of metabolites associated with treatable diseases (5, 6). We previously developed an ESI-MS/MS assay for MPS-I using an enzyme substrate obtained from the degradation of commercially available heparin (3). Although this assay works well, scale-up of the synthesis of the substrate has proven to be problematic because of technical difficulties in handling large volumes of nitrous acid for heparin degradation and removing impurities from the final reagents. We report here the development of a new and improved ESI-MS/MS assay that directly measures the reaction velocity of IdA in rehydrated dried blood spots (DBS) and may be adapted for the newborn screening of MPS-I. This assay makes use of a modified substrate that can be prepared by total synthesis on a scale suitable for worldwide newborn screening (10 g of substrate for more than 1 million assays).

All experiments were conducted in compliance with institutional review board guidelines. In all affected patients, MPS-I had been diagnosed previously with established clinical and biochemical procedures. DBS were kept at ambient temperature during shipment (<10 days) and then stored at -20°C in ziplock

⁴ Nonstandard abbreviations: MPS-I, mucopolysaccharidosis type I; IdA, α -L-iduronidase; ESI-MS/MS, electrospray ionization-tandem mass spectrometry; DBS, dried blood spots; IdA-S, IdA substrate; IdA-IS, IdA internal standard; IdA-P, IdA product.

plastic bags (one bag sealed inside a second bag). Ziplock bags were kept in a sealed plastic box containing desiccant (anhydrous CaSO_4 granules). A description of the method used for the synthesis of the IdA substrate (IdA-S) and the IdA internal standard (IdA-IS) is provided in the Data Supplement that accompanies the online version of this Brief Communication at <http://www.clinchem.org/content/vol54/issue12>.

A single 3-mm diameter DBS (containing approximately 3.6 μL of blood) was obtained with a leather punch and was placed in a 1-mL Eppendorf tube. Extraction buffer [160 μL of 0.1 mol/L sodium formate, pH 3.4, containing 75 $\mu\text{mol/L}$ D-saccharic acid 1,4-lactone (Sigma); storage at -20°C] was added to the tube. After vortex-mixing for 1 min, the tube was rocked gently on an orbital shaker for 45 min at 37°C . We transferred 20 μL of this blood extract to a 0.6-mL Eppendorf tube, and 10 μL of 1.5 mmol/L IdA-S in water (stored at -20°C) was added. The enzymatic reaction was incubated for 20 h at 37°C in a thermostated-air shaker and then was quenched by adding 100 μL of 0.1 mol/L sodium acetate, pH 5.4. We then added 10 μL of 20 $\mu\text{mol/L}$ IdA-IS in water (stored at -20°C), and the tube was vortex-mixed. A blank was prepared by incubating a tube with 20 μL of blood extract and a tube with 10 μL of 1.5 mmol/L IdA-S in 0.1 mol/L sodium formate, pH 3.4, separately at 37°C for 20 h, followed by mixing the 2 solutions together and adding 100 μL of 0.1 mol/L sodium acetate, pH 5.4, and 10 μL of 20 $\mu\text{mol/L}$ IdA-IS in water.

Extraction of IdA product (IdA-P) and IdA-IS from the enzymatic reaction and blank was performed by addition of 250 μL of ethyl acetate. After vortex-mixing for 15 s, the tube was centrifuged for 1 min with a table-top centrifuge at maximum speed. A 200 μL aliquot of the top organic layer was transferred to a 1-mL Eppendorf tube. The solvent was removed under a stream of nitrogen (typically 30 min at room temperature). To the residue we added 70 μL of 5 mmol/L ammonium formate in methanol, and the sample was transferred into a well of a 96-well plate (Greiner Bio-One, cat. #651201) for the Waters sample manager. A 20- μL aliquot of the sample was infused into the mass spectrometer and analyzed within 1 min of infusion.

ESI-MS/MS was carried out on a Waters ACQUITY tandem quadrupole instrument operating in positive-ion, multiple-reaction-monitoring mode (see online Supplemental Data). The parent ions for IdA-P and IdA-IS (m/z 391.2 and 377.2, respectively) were isolated and subjected to collision-induced dissociation. The fragment ions analyzed were m/z 291.1 and 277.1 derived from IdA-P and IdA-IS, respectively, by elimination of isobutene and carbon dioxide. The amount of product was calculated by comparing the ion peak intensities of IdA-P with IdA-IS.

We used a 13-step process with inexpensive starting materials to prepare IdA-S. It consists of an umbelliferyl- α -L-iduronide to which is attached a 4-carbon chain terminated by a *t*-butyl carbamylated amino group (Fig. 1A). Incubation with IdA present in DBS leads to enzymatic release of the iduronyl group to produce the umbelliferyl derivative product IdA-P (Fig. 1A). IdA-IS is closely related to IdA-P, but the IdA-IS carbon chain is shorter by 1 methylene group, so IdA-IS has a different molecular weight. ESI-MS/MS enables separate detection and quantification of IdA-P and IdA-IS by their fragment ions, after collision-induced elimination of the *t*-butylcarbamate group (100-Da mass difference; Fig. 1A).

To remove buffer salts, which are present in relatively high concentrations, we used a simple liquid-liquid extraction step that is appropriate for high-throughput analyses to extract IdA-P and IdA-IS. The presence of a charged carboxylate function on the sugar part of IdA-S at pH 5.4 prevents its extraction into the organic layer, whereas IdA-P and IdA-IS are not charged at pH 5.4 and are extracted into ethyl acetate. This extraction step is important because cleavage of the glycosidic bond of IdA-S during ESI-MS/MS can occur, forming IdA-P ions and thus giving rise to false-positive IdA activity. The MS/MS signal generated by the assay of a normal DBS is generally 4–10 times greater than the blank prepared under the conditions mentioned previously.

Assay optimization showed maximum IdA activity at pH 3.4 (see online Supplemental Fig. 1). The amount of IdA-P increases linearly with reaction time from 0–30 h (see online Supplemental Fig. 2); we chose 20 h for the standard incubation time for all subsequent assays. The amount of IdA-P formed at 20 h increased in a hyperbolic fashion as the concentration of IdA-S increased from 0–0.67 mmol/L, and K_m was determined to be 0.2 mmol/L (see online Supplemental Fig. 3). An IdA-S concentration of 0.5 mmol/L was chosen to be under saturation conditions. The amount of IdA-P increased with the surface area of added DBS (see online Supplemental Fig. 4), with a plateau reached at higher blood amounts (presumably due to the presence of endogenous inhibitors in the DBS). We thus chose to use a 3-mm DBS punch.

Previously published data showed that IdA in DBS is stable for at least 4 years (5). IdA activity in 5 patients (range, 0–0.268; mean, 0.053 $\mu\text{mol/h/L}$ blood) was well below the range of activity in samples obtained from 10 unaffected newborns (range, 7.4–23.4; mean, 12.3 $\mu\text{mol/h/L}$ blood) (Fig. 1B). IdA activity in the 5 MPS-I carriers was intermediate (range, 1.4–5.6; mean, 2.9 $\mu\text{mol/h/L}$ blood), but still well separated from the activities from the affected patients. Assay imprecision was calculated by replicate analyses of the

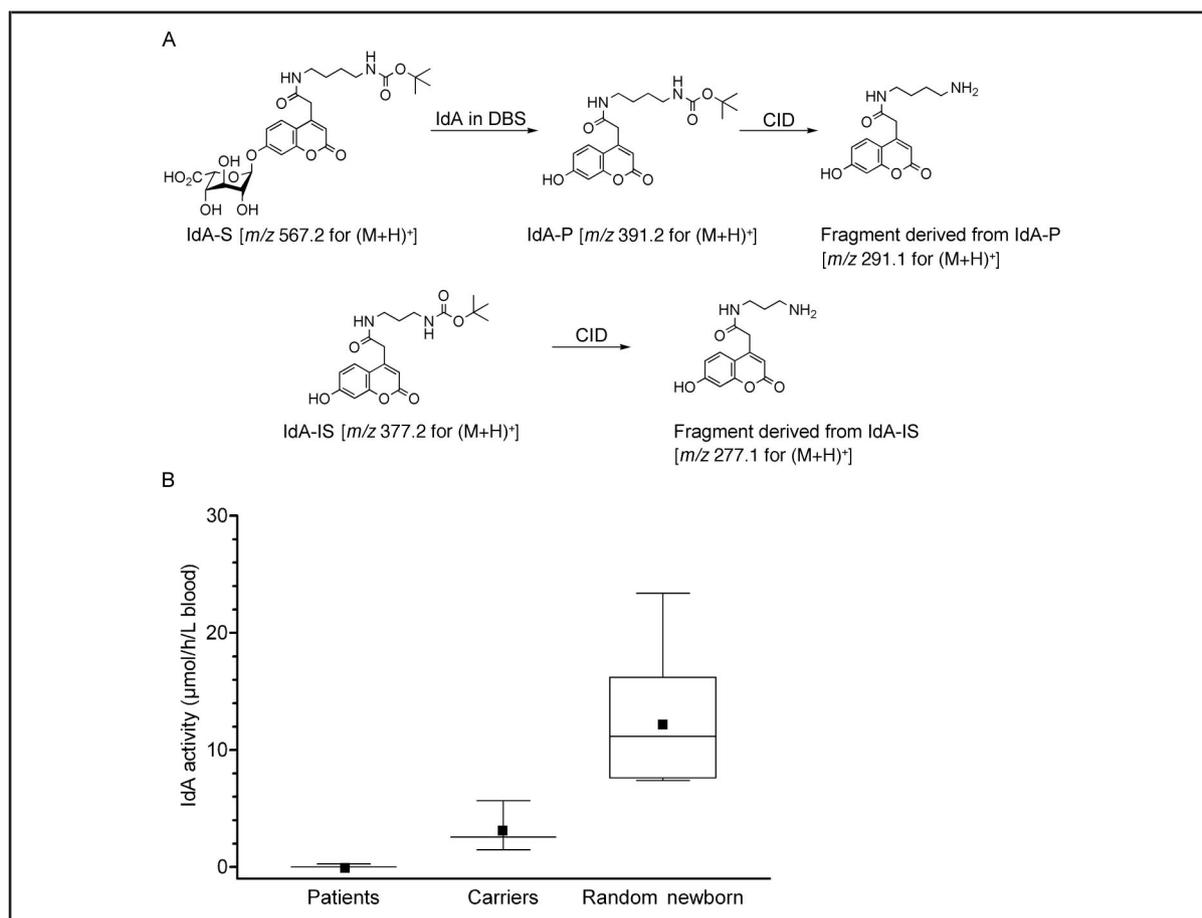


Fig. 1. IdA reaction measured in this study (A), and range of IdA activities in DBS (B).

(A), the structures of IdA-S, IdA-P, and IdA-IS are shown. The structures of the fragment ions derived from IdA-P and IdA-IS after collision-induced dissociation (CID) in the mass spectrometer are also shown. IdA-P and IdA-IS were quantified by ESI-MS/MS in positive-ion multiple-reaction-monitoring mode using the (M+H)⁺ ion. (B), IdA activities were measured in DBS by the standard assay described in the text. The horizontal bars indicate the full range of values, the box indicates the 25%–75% values, the horizontal line inside the box indicates the median, and ■ indicates the mean. IdA activity values for each sample are given in Table 1 of the online Data Supplement. Data are for 5 MPS-I patients, 5 carriers, and 10 unaffected newborns.

DBS from a healthy control; the within-assay CV was 5.9% ($n = 3$), and the interassay CV was 9.3% ($n = 10$).

The new ESI-MS/MS-based assay for IdA that we have developed should be practical for high-throughput analysis in newborn-screening laboratories and compatible with simultaneous assays of other lysosomal enzymes, including those for which ESI-MS/MS-based assays have already been developed (7, 8). The IdA assay can also be carried out by fluorometry. The substrate and internal standard used in this new assay overcome the synthetic problems encountered previously with the first IdA assay developed by our group. Each IdA assay requires only a small fraction of 3-mm DBS punch, 8.5 μg of substrate, and 0.075 μg of internal standard. Also, the pre-ESI-MS/MS purifica-

tion protocol used in this new assay is a simple liquid-liquid extraction and is easier to execute than the purification using C₁₈-silica plates reported in the previous assay (3). Scale-up synthesis of the new MPS-I substrate performed by workers at Genzyme has proceeded well. Studies to extend the ESI-MS/MS assay method to MPS-II and MPS-VI are ongoing.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors' Disclosures of Potential Conflicts of Interest: Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:

Employment or Leadership: None declared.

Consultant or Advisory Role: C.R. Scott, Genzyme; M.H. Gelb, Genzyme.

Stock Ownership: None declared.

Honoraria: C.R. Scott, Genzyme.

Research Funding: This work was supported by grants from NIH

(DK67859) and from Genzyme. C.R. Scott, NIH, Genzyme, and Biomarin; M.H. Gelb, NIH.

Expert Testimony: None declared.

Other: F. Turecek, travel reimbursement.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, preparation or approval of manuscript.

Acknowledgments: We are grateful to Joan Keutzer (Genzyme) for helpful discussions.

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DOI: 10.1373/clinchem.2008.115410
