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

Sophie Blanchard¹, Martin Sadilek¹, C. Ronald Scott², Frantisek Turecek^{1,*}, and Michael H. Gelb^{1,3,*}

¹Department of Chemistry, University of Washington, Seattle, Washington

²Department of Pediatrics, University of Washington, Seattle, Washington

³Department of Biochemistry, University of Washington, Seattle, Washington

Abstract

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. 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 prior to 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 intra-assay 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 intra-assay imprecision were less than 10 %. The synthesis of the α -L-iduronidase substrate is practical 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. It presents advantages compared to the one previously published from this laboratory and has the potential to be performed in a multiplex fashion with several lysosomal enzymes relevant to treatable lysosomal storage diseases.

Mucopolysaccharidosis type I (MPS-I) is a lysosomal storage disorder caused by the deficiency of α -L-iduronidase (IdA; EC 3.2.1.76) activity and can manifest three major clinical phenotypes: Hurler, Scheie, and Hurler-Scheie syndromes. IdA is essential for the degradation of the glycosaminoglycans dermatan and heparan sulfate within lysosomes. As symptoms may not be recognized early in life, the diagnosis of MPS-I is a challenging task. Enzyme replacement therapy and bone transplantation 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, the need for developing screens for the early recognition of MPS-I is of great interest. Fluorometric, radiometric, and electrospray ionization tandem mass

*Address correspondence to M. H. G. at: Departments of Chemistry and Biochemistry, University of Washington, Campus Box 351700, Seattle, Washington 98195; fax 206-685-8665, e-mail gelb@chem.washington.edu; or to F. T. at: Department of Chemistry, University of Washington, Campus Box 351700, Seattle, Washington 98195; fax 206-685-8665, e-mail turecek@chem.washington.edu.

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spectrometry (ESI-MSMS) assays have been developed (3,4). The latter offers the capability of assaying the products of several enzymes simultaneously (multiplexing).

Tandem MS is used in newborn screening programs to quantify the level of metabolites associated with treatable diseases (5,6). We previously developed an ESI-MSMS 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-MSMS assay that directly measures the reaction velocity of IdA in rehydrated dried blood spots (DBS) that may be adapted for the newborn screening of MPS-I, and that 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. All MPS-I affected patients 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 zip-lock plastic bags (one bag sealed inside a second bag). Zip-lock bags were kept in a sealed plastic box containing desiccant (anhydrous CaSO₄ granules). A description of the method used for the synthesis of the iduronidase substrate (IdA-S) and the iduronidase internal standard (IdA-IS) is provided in the Supplemental Data.

A single 3-mm diameter DBS (containing ~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 µmol/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. Twenty microliters of this blood extract was transferred 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 hours 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. Ten microliters of 20 µmol/L IdA-IS in water (stored at -20 °C) was then added, 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 hours, followed by mixing the two solutions together and adding 100 µL of 0.1 mol/L sodium acetate pH 5.4 and 10 µL of 20 µmol/L IdA-IS in water.

Extraction of 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 using 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 was 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 one minute of infusion.

ESI-MSMS 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 are *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.

IdA-S was prepared from inexpensive starting materials in 13 steps. It consists of an umbelliferyl- α -L-iduronide to which is attached a four-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). The internal standard IdA-IS is closely related to IdA-P but its carbon chain is shorter by one methylene group so that the internal standard has a different molecular weight. IdA-P and IdA-IS are separately detected and quantified by ESI-MSMS as 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 the product and internal standard. 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 as cleavage of the glycosidic bond of IdA-S during ESI-MSMS can occur, forming IdA-P ions and thus giving rise to false-positive IdA activity. The MSMS signal generated by the assay of a normal DBS is generally 4 to 10 times greater than the blank prepared in the conditions mentioned previously.

Assay optimization showed maximum IdA activity at pH 3.4 (See Supplemental Data Figure 1). The amount of IdA-P increases linearly with reaction time from 0 to 30 h (See Supplemental Data Figure 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 to 0.67 mmol/L, and K_m was determined to be 0.2 mmol/L (See Supplemental Data Figure 3). An IdA-S concentration of 0.5 mmol/L was chosen in order to be under saturation conditions. The amount of IdA-P increases with the surface area of added DBS (See Supplemental Data Figure 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.

Previous published data showed that IdA in DBS is stable for at least 4 years (5). As shown on Fig. 1B, IdA activity in 5 patients (range, 0-0.268; mean, 0.053 μ 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 μ mol/h/L blood). IdA activity in the 5 MPS-I carriers was intermediate (range, 1.4-5.6; mean, 2.9 μ mol/h/L blood), but still well separated from the activities from the affected patients. Assay imprecision was calculated by replicate analysis of the 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-MSMS-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-MSMS-based assays have already been developed (7,8). 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 8.5 μ g of substrate and 0.075 μ g of internal standard. Also, the pre-ESI-MSMS purification 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 has proceeded well by workers at Genzyme Corp. Studies to extend the ESI-MSMS assay method to MPS-II and MPS-VI are ongoing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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 are quantified by ESI-MSMS in multiple-reaction monitoring. (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.