

search. By identifying the gaps in their knowledge, residents were able to build a knowledge base and a set of resources for quickly answering difficult problems presented by their patients. Questionnaires revealed that residents who used the online database felt more comfortable with their abilities to succeed as lifetime learners than did residents at schools without such databases. Online tools are also used in otolaryngology, emergency medicine, and thoracic surgery residency programs (5–8). The database described herein similarly empowers clinical pathology residents and training programs.

In summary, we have developed an online database that is accessible from any computer with Internet access. This system can be used to document resident competency and simplify consultations with clinicians, improving patient care and laboratory service. As we have demonstrated, it can also be used to guide improvements in resident education. Above all, this online database will facilitate compliance with ACGME recommendations aimed at providing proficient physicians for the next century. Our future goals for the database are to expand its use by making it freely available to other institution, and to continue to study its impact on resident education and clinical service.

We thank the Laboratory Medicine residents for their contributions to the database from 2004–2006 and Dr. Petrie Rainey for many helpful discussions.

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Tandem Mass Spectrometry for the Direct Assay of Enzymes in Dried Blood Spots: Application to Newborn Screening for Mucopolysaccharidosis II (Hunter Disease), Ding Wang,¹ Tim Wood,² Martin Sadilek,¹ C. Ronald Scott,³ Frantisek Turecek,^{1*} and Michael H. Gelb^{1,4*} (Departments of ¹Chemistry, ³Pediatrics, and ⁴Biochemistry, University of Washington, Seattle, Washington; ²Biochemical Genetics Laboratory, Greenwood Genetic Center, Greenwood, South Carolina; *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; 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)

Background: A treatment for mucopolysaccharidosis II (Hunter syndrome) has recently become available. Therefore, we developed a high-throughput assay method appropriate for newborn screening for the relevant enzyme, iduronate 2-sulfatase.

Methods: We synthesized a new iduronate 2-sulfatase 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.

Results: When the assay was tested on dried blood spots, the iduronate 2-sulfatase activity measured for 13 patients with Hunter syndrome was well below the interval found for 57 randomly chosen newborns. The assay was more sensitive than previously reported iduronate 2-sulfatase assays.

Conclusions: This newly developed tandem mass spectrometry assay has the potential to be adopted for newborn screening of Hunter syndrome. This method also has the potential to be carried out in multiplex fashion to assay several different enzymes relevant to lysosomal storage diseases that are assayed in a single infusion into the mass spectrometer.

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We have been developing a panel of tandem mass spectrometry (MS/MS) assays of enzymes in dried blood spots (DBSs) for potential application to newborn screening of lysosomal storage diseases. Chamoles and coworkers showed that many lysosomal enzymes are active in rehydrated DBSs (1). The method involves addition of a designed, synthetic substrate for the selected enzyme to a buffer-rehydrated punch from a DBS. After incubation, the amount of enzyme-generated product is quantified, along with an isotope-labeled internal standard, by selective detection with electrospray ionization MS/MS (ESI-MS/MS). MS offers the advantages of analytical sensitivity, selectivity, and speed and is also ideally set up for multiplex analysis, whereby the products of many different enzymes may be quantified during a single infusion into the instrument. We previously developed a multiplex

MS/MS assay with DBSs that may be applied for newborn screening of Fabry, Gaucher, Hurler, Krabbe, Niemann–Pick A/B, and Pompe diseases (2–4). In this report, we describe a new assay for mucopolysaccharidosis II (MPS-II) (Hunter disease) that uses MS/MS and DBSs. This assay has the potential to be multiplexable with the other assays noted above and also is suitable for newborn screening. Hunter disease is caused by deficiency of iduronate 2-sulfatase (IDS) (EC 3.1.6.13), a lysosomal enzyme required for the degradation of dermatan and heparan sulfate. Our method is timely because of the recent progress made in the development of treatment for Hunter disease by enzyme replacement (5).

All experiments were conducted in compliance with Institutional Review Board review. All affected patients had MPS-II that was previously diagnosed with established clinical and biochemical procedures. DBSs were kept at ambient temperature during shipment (<10 days) and then stored at -20°C in 2 zip-lock plastic bags (1 bag sealed inside of a 2nd bag). Zip-lock 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 iduronate 2-sulfatase substrate (IDS-S) from commercially available heparin and the iduronate 2-sulfatase internal standard (IDS-IS), along with structures of the materials, is provided in the Data Supplement that accompanies the online version of this technical brief at <http://www.clinchem.org/content/vol53/issue1>.

A single 2-mm DBS punch (containing $\sim 1.6\ \mu\text{L}$ of blood) was obtained with a standard leather punch and was placed into a single well of a 96-well plate (F96 MaxiSorp Nunc-Immuno Plate, Nunc Inc., cat. no. 442404), followed by addition of $24\ \mu\text{L}$ of 16 mmol/L lead(II) acetate aqueous solution (6).

The plate was capped with a Teflon-lined cover (Cap Mats, E&K Scientific, cat. no. EK 99116) and gently shaken on an orbital shaker for 10 min at room temperature. To the same well were added $12\ \mu\text{L}$ of 0.33 mol/L sodium acetate buffer (pH 4.5) and $4\ \mu\text{L}$ of 1 mmol/L IDS-S in water (stored at -20°C); the plate was capped and gently shaken for 20 h at 37°C in a thermostated air shaker. The reaction was quenched by addition of $100\ \mu\text{L}$ of 1 mmol/L Na_2HPO_4 buffer, pH 7.85. IDS-IS was added ($20\ \mu\text{L}$ of 25 $\mu\text{mol/L}$ in water, stored at -20°C). The solution was mixed by use of a multichannel pipettor to pipette the liquid up and down a few times; then the liquid was transferred to a well of a 96-well filter plate (Innovative Microplate, cat. no. F20005) containing C18-silica bulk medium (see below).

The filter plate was placed on a vacuum manifold system (Millipore Inc., cat. no. MAVM0960R) attached to a water aspirator. After sample loading, 3.2 mL of H_2O was added to wash the solid phase. The manifold was charged with a deep-well receiver plate (96-well megatiter collective plates, CLP Inc., cat. no. 2045), and iduronate 2-sulfatase product (IDS-P) and IDS-IS were eluted with one $400\text{-}\mu\text{L}$ portion of 20% aqueous acetonitrile. Solvent was removed from the receiver with a vacuum desiccator under reduced pressure (~ 2 h at room temperature). To

each well was added $100\ \mu\text{L}$ of 5 mmol/L ammonium formate in methanol, and the sample was transferred to a small glass sample vial insert (Agilent, cat. no. 5183-2085), which was placed in a standard glass sample vial for the Agilent autosampler for infusion of a $5\text{-}\mu\text{L}$ aliquot into the mass spectrometer (see the online Data Supplement).

The 96-well filter plate was charged with C18-silica as follows. We suspended C18-silica bulk medium (2.0 g; Aldrich, cat. no. 377635) in a mixture of 7 mL of methanol and 3 mL of 3 g/L carboxymethyl cellulose sodium salt (Sigma, cat. no. C5678) aqueous solution. With constant stirring by magnetic bar, a 0.7-mL portion of slurry was added to each well. Solvent was removed with a vacuum filter manifold, and the solid phase was washed with 1.5 mL of H_2O before use.

ESI-MS/MS was carried out on an Applied Biosystems API-4000 tandem quadrupole instrument operating in negative multiple reaction-monitoring mode. Instrument settings are provided in the online Data Supplement. The precursor ions for IDS-P and IDS-IS were m/z 677 and 682, respectively, and the product ions were m/z 597 and 602 for IDS-P and IDS-IS, respectively (see Fig. 1 in the online Data Supplement). The amount of product was calculated by comparing ion peak intensity of IDS-P and IDS-IS.

Incubation of IDS-S with IDS present in the rehydrated DBSs leads to enzymatic release of the 2-sulfate group on the iduronyl residue to produce the product IDS-P. IDS-IS is chemically identical to IDS-P but is 5 Da heavier owing to the presence of 5 deuterium atoms in the benzoyl group. ESI-MS/MS detects and quantifies IDS-P and IDS-IS product ions after collision-induced elimination of SO_3 from the anhydromannosyl residue (80-Da mass difference; see Fig. 1 in the online Data Supplement). Our design of IDS-S was based on earlier work showing that the 6-sulfate group present in the 2,5-anhydromannosyl residue leads to a more efficient substrate for IDS (6). IDS-S also contains a hydrophobic 1,7-diaminoheptyl linker capped with a benzoyl group. The former provides a hydrophobic moiety that allows for sample clean-up before ESI-MS/MS, and the latter provides an additional hydrophobic moiety and a practical and inexpensive heavy isotope tag.

Although ESI-MS/MS provides for very selective detection of IDS-P and IDS-IS in the presence of a large number of other metabolites present in the DBSs, it is still necessary to remove the buffer salts before ESI-MS/MS because they are present in relatively high concentration and could cause interference during the ionization process. Because IDS-P is a relatively polar molecule, liquid-liquid extraction with organic solvent is not feasible, and thus we resorted to solid-phase extraction by use of reversed-phase. The presence of the hydrophobic benzoylated 1,7-diaminoheptyl linker of IDS-P and IDS-IS allows these compounds to be retained on reversed-phase C18-silica gel, whereas the buffer salts pass through during the wash with H_2O . IDS-S is more polar than IDS-P and is washed off the resin with the buffer salts. This is important because some cleavage of IDS-S during ESI-MS/MS

can occur, forming IDS-P ions and thus giving rise to false-positive IDS activity. IDS-P and IDS-IS are fully retained on the resin under these conditions and are fully eluted with 20% acetonitrile in water (data not shown).

As shown in Fig. 2 in the online Data Supplement, the amount of IDS-P increases with reaction time from 0–36 h. We choose 20 h for the standard incubation time for all subsequent assays. As shown in Fig. 3 in the online Data Supplement, the amount of IDS-P formed at 20 h increases in a hyperbolic fashion as the concentration of IDS-S is increased from 0 to 0.3 mmol/L [mean (SD) $K_m = 0.2$ (0.03) mmol/L; $V_{max} = 35$ (2) $\mu\text{mol h}^{-1}$ (L of blood) $^{-1}$]. An IDS-S concentration of 0.1 mmol/L was chosen for all subsequent assays. The amount of IDS-P increases with the surface area of added DBSs (see Fig. 4 in the online Data Supplement), with a plateau reached at higher blood amounts (presumably due to the presence of endogenous inhibitors in the DBSs). On the basis of these data, we chose to use a 2-mm DBS punch.

Analysis of IDS activity in DBSs obtained from 57 randomly selected newborns, as well as DBSs obtained from 13 MPS-II patients (Fig. 1), clearly shows that IDS activity in the patients [interval, 0.044–1.9; mean, 0.809; median, 0.636 $\mu\text{mol h}^{-1}$ (L of blood) $^{-1}$] is well below the activity interval seen for the samples obtained from the 57 randomly newborns [interval, 10.4–83.4; mean, 36.4; median, 34.2 $\mu\text{mol h}^{-1}$ (L of blood) $^{-1}$]. Values for individual IDS are listed in Table 1 in the online Data Supplement. The activity in control experiments (all components but DBS replaced with a 2-mm punch of filter paper without blood, with incubation) of 2.89 was subtracted from all measurements. In other controls, we obtained a value of 1.96 when all components were present, but the reaction quenched immediately, and the value was 2.07 when all components except IDS-S were incubated. The same data plotted according to percentile rankings are shown in Fig. 5 in the online Data Supplement. We did not carry out a systematic study of the IDS activity as a function of the storage time of the DBS; however, analysis of DBSs collected in 2004 gave an activity in the reference interval.

The IDS assay we have developed is expected to be compatible with simultaneous assays of numerous lysosomal enzymes, including the 6 for which ESI-MS/MS-based assays have been developed (2–4). Note that IDS-P and the substrate for α -L-iduronidase (for assay of MPS-I) (4) are structurally similar, but because they differ in the number of methylene groups in the hydrophobic linker, it should be straightforward to analyze for MPS-I and -II in the same infusion into the mass spectrometer. Other assays for IDS are the radiometric procedure that uses a tritiated disaccharide and ion exchange chromatography to separate sulfated substrate from nonsulfated product (6), and a recently described fluorometric assay that makes use of added rabbit liver α -L-iduronidase (purified from rabbit liver), which liberates 4-methylumbelliferone only after IDS-catalyzed desulfation (7). These assays are simple, but the ESI-MS/MS assay uniquely offers the capability of multiplexing with other enzyme assays. The sensitivity of the radiometric and fluorometric assays

cannot be estimated from the published results because the signal from the minus enzyme control was not reported. For the ESI-MS/MS assay, the assay signal is typically \sim 25-fold higher than the signal for the control carried out with a 2-mm filter paper punch that lacks blood, and this result was obtained by infusing only 1/20th of the assay mixture into the mass spectrometer.

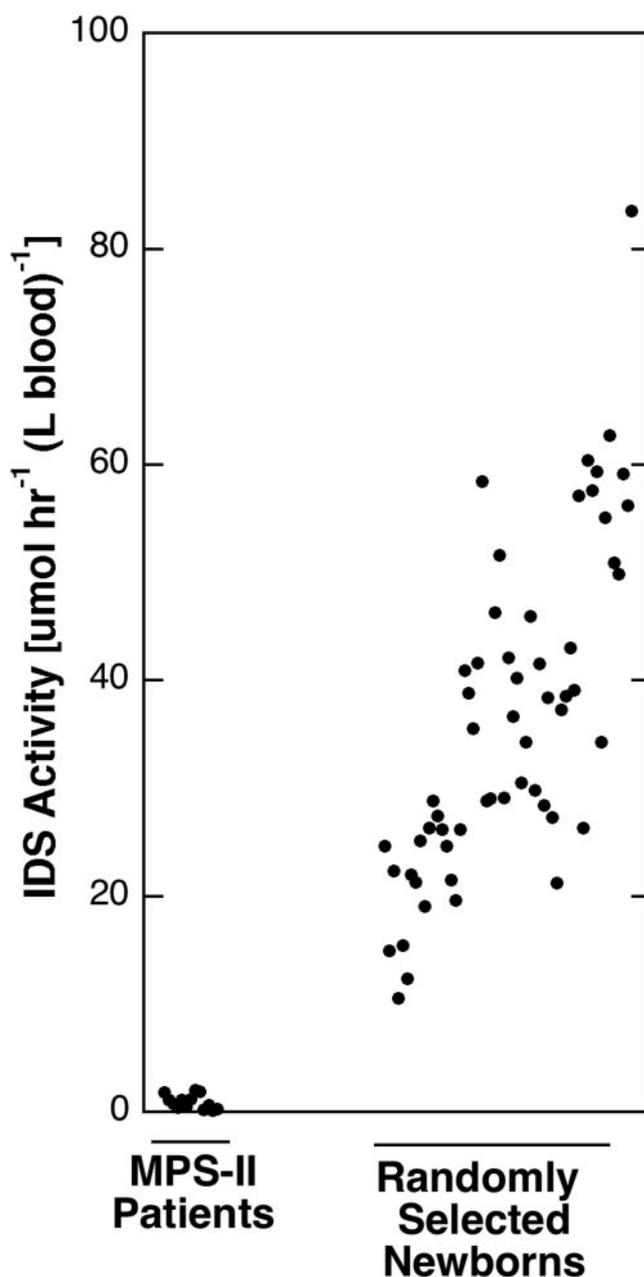


Fig. 1. IDS activities measured in DBSs by the standard assay for 13 MPS-II patients and 57 randomly chosen newborns.

MPS-II patients: mean, 0.81; median, 0.64; SD, 0.66. Randomly selected newborns: mean, 36.3; median, 34.2; SD, 15.1. The ages of the MPS-II patients at the time DBS samples were obtained were 1–5 years ($n = 5$), 6–10 years ($n = 5$), and >11 years ($n = 3$).

This work was supported by a grant from the National Institutes of Health (DK67859) and from Genzyme Inc. We are grateful to Dr. Joan Keutzer (Genzyme Inc.) for helpful discussions.

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Previously published online at DOI: 10.1373/clinchem.2006.077263
