Improved Reagents for Newborn Screening of Mucopolysaccharidosis Types I, II, and VI by Tandem Mass Spectrometry

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Supporting Information

ABSTRACT: Tandem mass spectrometry for the multiplex and quantitative analysis of enzyme activities in dried blood spots on newborn screening cards has emerged as a powerful technique for early assessment of lysosomal storage diseases. Here we report the design and process-scale synthesis of substrates for the enzymes α-L-iduronidase, iduronate-2-sulfatase, and N-acetylgalactosamine-4-sulfatase that are used for newborn screening of mucopolysaccharidosis types I, II, and VI. The products contain a bisamide unit that is hypothesized to readily protonate in the gas phase, which improves detection sensitivity by tandem mass spectrometry. The products contain a benzoyl group, which provides a useful site for inexpensive deuteration, thus facilitating the preparation of internal standards for the accurate quantification of enzymatic products. Finally, the reagents are designed with ease of synthesis in mind, thus permitting scale-up preparation to support worldwide newborn screening of lysosomal storage diseases. The new reagents provide the most sensitive assay for the three lysosomal enzymes reported to date as shown by their performance in reactions using dried blood spots as the enzyme source. Also, the ratio of assay signal to that measured in the absence of blood (background) is superior to all previously reported mucopolysaccharidosis types I, II, and VI assays.

Newborn screening of a subset of lysosomal storage diseases is an important new area of laboratory medicine since treatments are available for these diseases, and early initiation of treatment often leads to a better clinical outcome.1,2 We have been developing the use of tandem mass spectrometry for the multiplex assay of several lysosomal enzymes in dried blood spots on newborn screening cards.3-8 The method allows for the quantification of enzymatically formed product by including an internal standard, which is structural similar to the enzymatic product or structurally identical but with heavy isotope substitution. The methodology has been provided in different formats for the sample preparation steps prior to mass spectrometry.9-18 The simplest solution that works well seems to be liquid–liquid extraction with organic solvent followed by flow injection into the tandem mass spectrometer.15,19

Reagents for tandem mass spectrometry analysis of six lysosomal enzymes are currently prepared by Genzyme Corporation and distributed by the Centers for Disease Control and Prevention. The latter has also developed standard, quality control dried blood spots, thus providing a consistent set of samples to be used by newborn screening laboratories worldwide.20 These reagents have been used in different pilot studies, which have helped to validate newborn screening for lysosomal storage diseases.19,21-24 (as well as unpublished studies in Taiwan and Illinois).

Scheme 1 shows the structure of the substrates developed by us previously for the tandem mass spectrometry assay of the enzymes relevant to Pompe disease and mucopolysaccharidosis-I (MPS-I).3,5 The MPS-I substrate contains the fluorogenic 7-hydroxycoumarin unit, thus allowing this reagent to be used for fluorometric assays of α-L-iduronidase (the enzyme relevant to MPS-I) in addition to tandem mass spectrometry. However, it is now clear that tandem mass spectrometry-based lysosomal enzyme analysis offers distinct advantages over fluorometric assays. First, the background signal seen in the tandem mass spectrometry assay when substrate is omitted is virtually zero (i.e., no components of blood other than enzymatic product are detected in the product mass spectrometry ion-selective channels), whereas nonproduct components of the blood contribute to significant autofluorescence in the fluorometric assay. This leads to a much higher assay-to-blank ratio for the tandem mass spectrometry assay.

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versus the fluorometric assay and to a much more accurate assessment of enzymatic activity in the low-end range. This in turn is expected to lead to a much lower rate of false positives in newborn screening of lysosomal storage diseases. Furthermore, there is no fluorometric assay reagent that leads to reliable newborn screening of Niemann–Pick type-A/B disease as available fluorometric reagents give a significant false negative rate. This is due to a prevalent pathogenic mutation in the population that leads to low enzymatic activity with the natural sphingomyelin substrate but a high activity with the artificial fluorometric substrate. The tandem mass spectrometry assay uses a substrate that is similar in structure to the natural sphingomyelin substrate but a high activity with the artificial fluorometric substrate. This tandem mass spectrometry assay uses a substrate that is similar in structure to the natural sphingomyelin substrate (the only difference is the length of the fatty acyl group attached to the sphingosine amino group); the false negative problem is not an issue with this technique.

The Pompe and MPS-I products are shown in Scheme 2. Recently, we have observed that the Pompe product gives an ~10-fold higher signal per mole in the tandem mass spectrometer compared to the MPS-I product. A detailed theoretical study suggests that the bisamide unit of the Pompe product allows for a particularly stable protonated ion species in the gas phase as shown in Scheme 2. This enhanced sensitivity and the lack of need for the fluorometric 7-hydroxycoumarin unit, we decided to prepare new substrates for MPS-I, -II, and -VI that contain the type of aglycone present in the Pompe reagent. These new reagents are also simpler to synthesize compared to the 7-hydroxycoumarin containing reagents. In the current study we present synthesis of the new reagents, show that they can be scaled up to amounts needed to support worldwide newborn screening, and show that they display enhanced tandem mass spectrometry performance compared to the original reagents.

### EXPERIMENTAL SECTION

**Materials.** Full details for the synthesis of all new reagents is given as Supporting Information. Patent applications have been filed with the U.S. Patent Office for the compounds described in this study. Buffers to assay MPS-I, -II, and -VI were as follows: MPS-I, 100 mM ammonium formate (Sigma), pH 4.4, 10 g/L sodium taurocholate (Sigma); MPS-II, MPS-I buffer plus 7.5 mM barium(II) acetate (Sigma), 5.0 mM cerium(III) acetate (Sigma); MPS-VI, MPS-II buffer plus 150 μM 2-acetamido-2-deoxy-glucosonic acid lactone (Santa Cruz Biotechnology). All dried blood spots were obtained with Institutional Review Board (IRB) approval at the University of Washington. Dried blood spots were stored at −10 to −20 °C in closed jars with desiccant.

**Enzymatic Assays.** MPS-I assays used a 3 mm dried blood spot punch in a 96-deep well plate (1 mL round-bottom Corning Costar, Fisher, cat. no. 09-761-116A). To the punch was added 30 μL of buffer containing 500 μM MPS-I substrate and 3.5 μM MPS-I internal standard. Substrate and internal standard were added to the punch to achieve the saturated concentration of substrate. The substrate was added to the punch in 10 μL aliquots at time zero, and at the times shown in Table 1.

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**Scheme 1**

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**Scheme 2**

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standard were added to a vial from stock solutions in methanol, the solvent was removed in a centrifugal evaporator (Speed-Vac), and assay buffer was added followed by vortex mixing to prepare the stock solution, which could be stored at −10 to −20 °C for at least a few months. The plate was sealed with sealing film (AxySeal, VWR International, cat. no. 10011-117)
for overnight (16 h) incubation at 37 °C with orbital shaking (250 rpm).

After incubation, 100 μL of a 1:1 mixture of ethyl acetate/methanol was added to each well with a multichannel pipet to quench the reaction. Additional 400 μL of ethyl acetate and 200 μL of water were added to each well, and mixing was achieved by pumping up and down with the pipettor 20 times over a few minutes. Plates were covered with polyester-based sealing film and centrifuged at 3000 rpm for 5 min at room temperature to separate the two liquid layers. Aliquots (200 μL) of the top liquid phase were removed from the plate and transferred to a new 96-well plate (0.5 mL, Axygen Scientific, VWR International, cat. no. 47743-982), the solvents were evaporated under a nitrogen stream at room temperature, and samples were reconstituted in 100 μL of the infusion solvent (80:20 methanol/water with 5 mM ammonium formate). The plates were carefully sealed with aluminum foil and subjected to flow injection tandem mass spectrometry on a Waters Xevo TQ instrument. Autosampler and mass spectrometer settings are given in Supporting Information.

MPS-II assays were carried out as above for MPS-I but with 1 mM MPS-II substrate and 5 μM MPS-II internal standard. After incubation, 200 μL of 44 mM citric acid in water was added to each well with a multichannel pipet to quench the reaction and to protonate the carboxylate of the iduronic acid residue of the product to allow extraction into organic solvent. Ethyl acetate (400 μL) and water (100 μL) were added to each well, and samples were mixed as above. Plates were additionally processed as for MPS-I.

MPS-VI assays were carried out as above but with 1 mM MPS-VI substrate and 5 μM MPS-VI internal standard. After incubation, two different work-up protocols were used. In one variation the reaction was quenched with 100 μL of 1:1 ethyl acetate/methanol. Additional 400 μL of ethyl acetate and 100 μL of water were added to each well, and plates were processed as for MPS-I assays. In the second variation, quenching of incubated samples was with 100 μL of a suspension of DEAE cellulose (Whatman DES5 preswollen, 16 mg of resin per 100 μL of water) followed by 400 μL of ethyl acetate. The samples were processed as above for the MPS-I assays.

**Extraction Studies.** The various enzyme products (300 pmol) were dissolved in 30 μL of assay buffer to give 10 μM analyte. The sample was processed by liquid—liquid extraction as above and submitted to flow injection tandem mass spectrometry as above. The amount of product detected by mass spectrometry was compared to that measured when 300 pmol of enzymatic product was directly injected into the instrument. The numbers were corrected for dilution factors to obtain the percent of total product added to assay buffer that was recovered in the organic phase.

**RESULTS AND DISCUSSION**

**Design and Synthesis of Reagents.** The synthesis of the new MPS-I substrate, MPS-II substrate, and MS-VI substrate is shown in Schemes 3 and 4. The aglycones were synthesized by a common pathway (Scheme 3). Commercially available diaminokanes were converted to monobenzoylated species 1a,b by treatment with methyl benzate.28,29 The resulting primary amine underwent a Michael addition with acrylamide 2, and the resulting secondary amine was acylated with the appropriate acyl chloride to give aglycones 3a−c in a one-pot series of reactions. Purification steps were either recrystallization or silica chromatography without the need for high-resolution separation. These aglycones have been prepared on the 100 g scale.

MPS-I substrate was made by condensing aglycone 3b with protected iduroyl-F 4− to give 5b. Deacetylation afforded 6b, and saponification afforded MPS-I substrate (Scheme 4). Aglycone 3b is the MPS-I product. MPS-I internal standard was prepared using a route similar to that used for MPS-I product but using commercially available and inexpensive pentadecaeteriobenzyl chloride (Supporting Information). It is chemically identical to MPS-I product but containing five deuteriums in the benzyl group.

MPS-II substrate was prepared from aglycone 3c (Scheme 3) by condensation with 4 (Scheme 4). After deacetylation, 6c was selectively sulfated on the 2-OH group by formation of the dibutyl stannylene acetal and treatment with SO3−Et3N as described.30 Saponification of the methyl ester afforded MPS-II substrate.

To prepare MPS-VI substrate, aglycone 3a was condensed with protected N-acetylglucosamine-1-Cl 7 followed by deacetylation to give MPS-VI product. The latter was selectively benzoylated on the 3- and 5-OH groups, the 4-OH was sulfated, and debenzoylation afforded the MPS-VI substrate (Scheme 4).

All of these new reagents contain the same bisamide unit found in the Pompe reagent and should form the stable protonated species shown in Scheme 2. The new reagents are also easier to prepare than the original reagents owing to the lack of the 7-hydroxyxoumarin unit. MPS-II and MPS-VI substrates contain longer acyl chains attached to the secondary amine of the aglycone compared to MPS-I substrate. This is because, prior to tandem mass spectrometry, assay mixtures are subjected to liquid—liquid extraction with ethyl acetate to remove buffer salts that would interfere with the electrospray ionization process. Since MPS-II and MPS-VI products contain a monosaccharide, we used longer acyl groups to drive the products into ethyl acetate. We measured the fraction of MPS-I, -II, and -VI products that are extracted into ethyl acetate by submitting the extracts to tandem mass spectrometry analysis compared to that of a standard solution. Extraction yields were found to be 70%, 61%, and 30% for MPS-I, -II, and -VI products, respectively. Incomplete extraction is tolerated since the internal standards will be extracted to the same extent as the corresponding products.

Tandem mass spectrometry analysis of MPS-I, -II, and -VI products show that all three give rise to a major fragment ion due to cleavage pathway shown in Scheme 5. Cleavage of the precursor ions along a major reaction pathway improves sensitivity of product detection as formation of multiple product ions would decrease the signal intensity since only a single ion reaction is monitored.

**Enzymatic Assays Using Dried Blood Spots.** The new substrates for MPS-I, MPS-II, and MPS-VI were tested side by side with our original reagents (see the introduction) using a 3 mm punch of a dried blood spot from a single adult as the enzyme source. After incubation, liquid—liquid extraction with ethyl acetate was used to extract enzymatic product and internal standard into a buffer salt-free, organic solvent phase since high salt interferes with the electrospray ionization process in the mass spectrometer. Internal standards, pentadecaeteriobenzyaled chloride, were used to convert the observed mass spectrometer signals into moles of product formed. Use of a chemically identical internal standard accounts for any losses of product due to many factors including absorption of analytes.
onto surfaces, incomplete extraction into organic solvent, consumption of products by downstream enzymes, and differential suppression of ion formation in the electrospray ionization source of the mass spectrometer.

Prior to tandem mass spectrometry (MS/MS), it is helpful to remove most of the substrate in the case of sulfatasases (MPS-II and -VI) since substrate-to-product conversion occurs to a small extent in the electrospray ionization source (due to local heating and possibly other factors). In the case of MPS-II, the reaction is quenched with citric acid to protonate the carboxylate of the iduronic acid group of the MPS-II product so that it is well-extracted into ethyl acetate. Essentially all of the MPS-II substrate remains in the aqueous layer since it bears a monoanionic sulfate ester that remains deprotonated (the conjugate base of an acid whose pKₐ is <0). In the case of MPS-VI, ethyl acetate extraction alone works to some extent to separate the charge-neutral MPS-VI product from MPS-VI substrate bearing a sulfate monoanion, but better results are obtained if the anion exchanger DEAE-cellulose is added during ethyl acetate extraction to tightly trap the substrate (see below). MPS-I substrate does not significantly fragment in the electrospray ionization source, and it is not necessary to reduce substrate amount prior to mass spectrometry. Also since the assay is not acidified prior to ethyl acetate extraction, most of MPS-I substrate remains in the aqueous phase.

Original and new reagents were used in side-by-side assays using multiple punches from the same dried blood spot (obtained from a healthy adult). Table 1 gives the observed ion counts for MPS-I, MPS-II, and MPS-VI products. The signal for the MPS-I product was 3.4-fold higher than that for an identical assay carried out with the original MPS-I reagent (7-hydroxycoumarin type, see the introduction). The sensitivity improvement factor for MPS-II product is 5.8. For MPS-VI, the assay gives a sensitivity enhancement of 7.9- and 8.9-fold, compared to the original reagent, if reaction quenching is with anion-exchange resin prior to liquid–liquid extraction into ethyl acetate or ethyl acetate extraction alone, respectively. These results support our hypothesis that enhanced protonation of the gas-phase product ions via the bisamide unit of the new reagents (Scheme 2) gives a larger ion count signal.

Table 2 gives the specific activities of the lysosomal enzymes for the original and new assay reagents. These are expressed as micromoles of product per hour per liter of blood. Internal standard ion counts are used to convert the observed product ion counts to moles of products. Each 3 mm dried blood spot punch contains the components of 3.3 μL of blood. We also carried out control assays in which a 3 mm filter paper punch (no blood) was included, and the sample was incubated and processed as for the complete assay. For MPS-I, the specific activities with the original and new reagents are similar, showing that both substrates are hydrolyzed with similar catalytic efficiencies. In the absence of blood, the new reagent gives a lower background signal compared to that from the original reagent. This presumably reflects a combination of lower amounts of new substrate extracted into ethyl acetate compared to the original substrate and/or lower break down of new substrate in the electrospray ionization source compared to the original substrate. As a result the blood to no-blood assay response is 5.6 times higher for the new versus original assay. For MPS-II, the specific activities measured with the original and new substrates are similar, and the blood to no-blood ratio is 2-fold higher with the new reagent. For MSP-VI the new reagent is a better substrate, with a specific activity that is about 8-fold higher than that with the original substrate. When the anion exchanger is used in the reaction quench the blood to no-blood ratio jumps from 4.9 to 97.4 when the original reagent is replaced with the new reagent. With the new reagent, this ratio is smaller, 16.7, when ethyl acetate extraction alone is used, suggesting that removing more substrate from the organic extract by trapping it on the anion-exchange resin leads to a decrease in background signal. This in turn suggests that most of the background comes from substrate-to-product conversion in the electrospray ionization source.

Table 3 gives the coefficient of variation (CV, %) for three injections of an identical solution into the mass spectrometer (intra-assay) or for three independent assays using three punches from the same dried blood spot (interassay) for assays with the original and new reagents. In all cases, CVs were <10%. These numbers are generally consistent with CVs for other assays used in newborn screening laboratories and illustrate the high reproducibility of these assays.

We measured the activities of the MPS-I, MPS-II, and MPS-VI enzymes in dried blood spots from 15 random newborns and from an affected child (diagnosis previously confirmed by standard methods). Figures 1–3 give a graphical description of the results, and all numerical values are provided in Supporting Information Tables 1–3. For MPS-I, -II, and -VI, all normals are well-separated from the affected patient. The ratio of mean normal activity to that of the affected child is 40 for MPS-I, 32 for MPS-II, and 17 for MPS-VI. The corresponding ratios
obtained by digital microfluidic fluorescent assays are 6.5 for MPS-I and 3.8 for MPS-II (no data for MPS-VI). The results show that the MS/MS assay greatly outperforms the fluorescence assay in terms of difference in activity between unaffected and lysosomal storage disease-affected individuals.

### CONCLUSIONS

The assay performance of the new reagents for MPS-I, -II, and -VI described in this study is superior to that obtained with our earlier reported reagents for assay of lysosomal enzymes in dried blood spots. The new reagents are easier to synthesize than the previous reagents and have been made on the multigram scale appropriate to support worldwide newborn screening. Enhanced product ion counts in the mass spectrometer are especially important for sulfatases (MPS-II and -VI) since these enzymes generate products containing a monosaccharide, which generally ionize poorer in the electro-spray source compared to products that lack a carbohydrate group (i.e., MPS-I). The ratio of complete assay to no-blood background assay response with the new reagents is superior to those reported in all previously described assays of these three lysosomal enzymes. This in turn should lead to a larger span in assay responses when blood samples from healthy individuals are compared to those from disease-affected newborns. These new MPS-I, -II, and -VI reagents are currently being used in large-scale pilot studies in newborn screening laboratories, and parameters including positive predictive values and false positive rates will be reported upon completion of these trials.

### Table 2. Specific Activities for Lysosomal Enzymes Using the New and Original Assay Reagents

| enzyme                | original reagent | new reagent |
|-----------------------|-----------------|-------------|
|                       | spec. act. (μmol/h/L) | spec. act. no blood (μmol/h/L) | ratio of complete assay to no-blood assay response | spec. act. (μmol/h/L) | spec. act. no blood (μmol/h/L) | ratio of complete assay to no-blood assay response |
| MPS-I                 | 1.57            | 0.03        | 52.3 | 1.21            | 0.004        | 293.5 |
| MPS-II                | 2.88            | 0.076       | 37.9 | 4.40            | 0.06         | 75   |
| MPS-VI (anion exch., EtOAc) | 1.41            | 0.28        | 4.91 | 12.17           | 0.125        | 97.4  |
| MPS-VI (EtOAc)        | 1.15            | 0.24        | 4.75 | 13.125          | 0.79         | 16.7  |

All specific activities are not blank-corrected.

### Table 3. Coefficient of Variation for the Lysosomal Assays

| enzyme                | original reagent | new reagent |
|-----------------------|-----------------|-------------|
|                       | intra-assay CV (%) | interassay CV (%) | intra-assay CV (%) | interassay CV (%) |
| MPS-I                 | 6.74            | 9.41        | 1.93 | 1.71 |
| MPS-II                | 1.90            | 5.56        | 8.47 | 8.65 |
| MPS-VI (anion exch., EtOAc) | 6.45            | 8.31        | 2.49 | 2.36 |
| MPS-VI (EtOAc)        | 4.94            | 5.30        | 3.28 | 5.15 |

Intra-assay CV was calculated from triplicate injections of an identical sample into the mass spectrometer. Interassay CV is calculated from three independent assays carried out with three different punches of the same dried blood spot.
ASSOCIATED CONTENT

Detailed synthetic procedures, spectroscopic data, and mass spectrometer instrument settings. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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