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Fluorimetric Assays for N-Acetylgalactosamine-6-Sulfatase and Arylsulfatase B Based on the Natural Substrates for Confirmation of Mucopolysaccharidoses Types IVA and VI

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Abstract

Background—Treatments have been developed for mucopolysaccharidoses-IVA (MPS-IVA) and MPS-VI suggesting the need for eventual newborn screening. Biochemical enzyme assays are important for diagnosis. Previously reported fluorimetric assays of the relevant enzymes are based on substrates with poor activity or specificity.

Methods—We developed new fluorimetric assays for N-acetylgalactosamine-6-sulfatase (GALNS) and arylsulfatase B (ARSB) based on the natural substrates, N-acetylgalactosamine-6-sulfate (and 4-sulfate), which have improved activity and specificity toward the relevant enzymes. The new substrates were tested on dried blood spots on newborn screening cards, and assays showed acceptable linearity in response with the amount of enzyme present (using quality control samples).

Results—When tested on dried blood spots from random newborns and affected patients, the assays showed good discrimination between the 2 sample groups.

Conclusions—The analytical range of the new fluorimetric assays, defined as the ratio of enzyme-dependent-to-enzyme-independent assay response, is likely to be insufficient to use these assays for newborn screening. Rather, these new fluorimetric assays should be useful in a diagnostic lab to confirm a diagnosis via biochemical enzyme testing.

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1. Introduction

There is interest in diagnosis of mucopolysaccharidosis types IVA (MPS-IVA, Morquio A syndrome) and MPS-VI (Maroteaux-Lamy syndrome) in part because of the availability of enzyme replacement therapy. The deficient enzymes are N-acetylgalactosamine-6-sulfatase (GALNS) and arylsulfatase B (ARSB). These enzymes contribute to breakdown of glycosaminoglycans in the lysosome. GALNS can be assayed fluorimetrically with the 4-methylumbelliferone (4MU) glycoside of galactose-6-sulfate in leukocyte lysates [1] and in dried blood spots (DBS) on newborn screening cards [2]. The 4MU fluorophore is released by β -galactosidase (human enzyme that is endogenous in leukocytes or supplemented with bacterial enzyme for DBS assays) only after the 6-sulfate is removed by GALNS. ARSB is assayed with the sulfatase substrate 4MU-sulfate [3] although this substrate is not specific for this enzyme; some selectivity is achieved by use of special buffers that favor ARSB activity over other sulfatases.

Recently, we have developed tandem mass spectrometry assays for GALNS and ARSB based on the natural enzyme substrates [4]. These assays are highly specific for each sulfatase and display much higher analytical ranges than the previously reported 4MU fluorimetric assays [4]. The analytical range is defined as the assay response due to the enzyme of interest divided by the non-enzymatic assay response. Assays with high analytical range are important for robust newborn screening where false positives are to be minimized and for accurate diagnoses where information about the disease severity is sought.

2. Materials and Methods

2.1. Materials

(Z)-Pugnac ((Z)-O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino N-phenylcarbamate) is from Santa Cruz Biotechnology. Ammonium formate, ammonium acetate, barium acetate, and cerium acetate were from Sigma. Quality control DBS were obtained from Dr. Jason Cournoyer, Perkin Elmer Life Sciences. Recombinant β -NGA was prepared by expression in *E. coli* as described [4]. Synthesis of 4MU-GalNAc-4-S and 4MU-GalNAc-6-S is given in Supplemental Material. DBS from random newborns were obtained from Prof. C. Auray-Blais, Univ. of Sherbrooke, Quebec. After DBS generation, they were stored for 1 week at ambient temperature and thereafter at -20°C for ~ 2 y. DBS from MPS patients were obtained with the help of the MPS Society. DBS were generated from patients and received at the Univ. of Washington within ~ 3 days, then stored at -20°C for up to 1 y. All human samples were handled according to our IRB protocol at the Univ. of Washington.

2.2. Fluorimetric assays

Assay buffer is 50 mmol/l ammonium acetate, 7.5 mmol/l $\text{Ba}(\text{OAc})_2$ and 5.0 mmol/l $\text{Ce}(\text{OAc})_3$, pH 5.0 (adjusted with acetic acid after metal salts added, stored 4°C). β -NGA buffer is 250 mmol/l bis-Tris-HCl, pH 7.5 (stored 4°C). Quench buffer is 0.5 M Na_2CO_3 adjusted to pH 10.7 using saturated NaHCO_3 . Assay cocktail was prepared by adding 4MU-

GalNAc-4-S or 4MU-GalNAc-6-S (10 mmol/l in water) and (Z)-Pugnac (18.9 mmol/l in methanol) to a tube, and solvent was completely removed in a vacuum centrifuge. Assay buffer was added to give 3 mmol/l substrate (ARSB) or 1 mmol/l (GALNS) and 0.5 mmol/l (Z)-Pugnac (mixed on a vortex mixer). Assay cocktail was prepared fresh before use.

In two wells of a 96 well polypropylene plate (Corning Cat. CLS3363), 3 mm DBS punches were incubated with 15 μ l of freshly prepared assay cocktail at 37°C in a sealed plate under constant shaking (250 rpm) for 16 h (plates sealed with adhesive film, Sigma). To one of the incubated wells, 16 μ l of bis-Tris buffer was added and to the other well 16 μ l of β -NGA (18.5 units/ μ l) in bis-Tris buffer was added, and the plate was sealed and incubated at 37°C for 2 h under constant shaking. Each well was quenched by addition of 200 μ l of quench buffer, and the plate was centrifuged (3000 rpm, 5 min). A portion of the supernatant (125 μ l) was transferred to a black 96-well plate (Perkin Elmer Life Sciences, Cat. 6005279). The plate was centrifuged again to remove bubbles (if necessary), and submitted to fluorimetry using a Perkin Elmer Victor³V plate reader with emission at 460 nm and excitation at 355 nm. A calibration curve was prepared as above using 4MU (0, 5, 10, 20 μ M, important to include DBS in the calibration because of quenching of fluorescence by blood components).

3. Results

3.1. New fluorimetric assays for GALNS

GALNS acts *in vivo* on both galactose-6-sulfate and N-acetylgalactosamine-6-sulfate residues of glycosaminoglycans. So far, a fluorimetric assay based only on 4MU-Gal-6-S has been reported [1, 2]. In the present study we explored 4MU-GalNAc-6-S as a potential substrate for GALNS for the first time. In our MS/MS study we found that hexosaminidase A present in DBS hydrolyzes the glycosidic linkage of GalNAc-6-sulfate conjugates, therefore it is necessary to include (Z)-Pugnac as an inhibitor of this enzyme so that 4MU release does not occur independently of GALNS. To release 4MU from the GALNS product we used the bacterial enzyme β -N-acetylgalactosaminidase (β -NGA) [5] since we showed in our mass spectrometry study that this enzyme does not act on the GALNS substrate 4MU-GalNAc-6-S and is not inhibited by (Z)-Pugnac [4].

The new GALNS fluorimetric assay makes use of two 3 mm DBS punches, which are incubated overnight. This was followed by a 2 h incubation with or without β -NGA. The GALNS-independent 4MU fluorimetric response is due to multiple factors: 1) Any 4MU present as an impurity in 4MU-GalNAc-6-S; 2) Any 4MU-GalNAc present in 4MU-GalNAc-6-S (4MU generated by added β -NGA); 3) Any desulfation of 4MU-GalNAc-6-S that occurs in buffer without DBS (4MU generated by β -NGA); 4) Any intrinsic fluorescence from 4MU-GalNAc-6-S; 5) Any fluorescence coming from the buffer or the DBS punch. By incubating 4MU-GalNAc-6-S with buffer containing β -NGA in the absence of the DBS punch we observed no measurable increase in fluorescence, which shows that 2 and 3 above are virtually zero. Also fluorescence due to 5 is <5% of the fluorescence due to 4 [4]. The amount of free 4MU in 4MU-GalNAc-6-S was found to be 0.016 % [4]. This was measured by the pH dependence of the fluorescence in the pH 3–10 range [4]. The analytical range for the assay was calculated as [(high quality control assay response in the plus β -NGA sample) - (high quality control assay response in the minus β -NGA sample)] divided

by (high quality control assay response in the minus β -NGA sample). Since elements 1, 4, 5 are present to the same extent in the plus and minus β -NGA samples, the subtraction in the numerator of the analytical range gives the net activity due to GALNS. The denominator of the analytical range is the assay response from elements that are independent of GALNS. Using the high quality control DBS, the assay response was found to be 6.5 (Table 1).

Execution of 5 independent GALNS assays with 4MU-GalNAc-6-S on the low, medium and high quality control DBS shows good linearity of the assay response with respect to the amount of GALNS in DBS and also provided the assay precision (expressed as the percent coefficient of variation) (Fig. 1 and Table 1). Values are reported as GALNS activity in units of $\mu\text{mol h}^{-1} (\text{L of blood})^{-1}$ obtained by subtracting the minus β -NGA assay response from that measured with β -NGA and using the fluorescence response of standard 4MU in buffer containing a DBS punch to convert the assay response to μmole of 4MU product. Inclusion of the DBS punch is critical as the 4MU fluorescence is quenched about 4-fold by components of the blood (data not shown). The Y-intercept in Fig. 1 is not at zero enzyme activity showing that the quality control base pool has finite GALNS. Fig. 2 shows the GALNS activities of 2 newborns and 2 MPS-IVA patients (individual values in Supplemental Table 1).

3.2. New fluorimetric assays for ARSB

The ARSB assay is similar to the GALNS assay except the substrate is 4MU-GalNAc-4-S. The hexosaminidase A inhibitor (Z)-Pugnac is also included since this enzyme acts on GalNAc residues containing the 4-sulfate [4]. 4MU is released by β -NGA only after the 4-sulfate is removed by ARSB. Activity and reproducibility data is shown in Table 1, and linearity data is shown in Fig. 1. Fig. 2 shows the ARSB activities of 51 healthy newborns and 3 MPS-VI patients (individual values in Supplemental Table 2).

4. Discussion

The new GALNS fluorimetric assay described here makes use of the preferred GalNAc-6-S substrate for GALNS together with (Z)-Pugnac and the secondary enzyme β -NGA. The latter is easily obtained by expression in *E. coli* [4] and does not add significantly to assay cost. Despite the improvement, the analytical range is relatively low at 6.5 compared to the recently developed tandem mass spectrometry assay for GALNS with a value of 198 (both values obtained with the same high quality control DBS) [4]. Studies show that this relatively low value is due mainly to the high intrinsic fluorescence of the 4MU substrate (element 4 described above) and not due to contamination by free 4MU [4]. Although the 4MU product is $\sim 2,000$ -fold more fluorescent per mole than the 4MU-substrate [4], the percent of substrate converted to product in the DBS assay is only 1–2%. This low value and the modest ability of this assay to distinguish between healthy random newborns and MPS-IVA patients (Figure 2) suggests that fluorimetric assays of GALNS will not be robust enough for newborn screening where a low level of false positives are required. The new GALNS assay may find its most use in leukocyte assays in a second-tier analysis that follows a screen positive results. However, even for this procedure, the tandem mass

spectrometry assay will provide more accurate GALNS activity data, which may be important to predict the severity of the disease.

The original assay for GALNS uses 4MU-Gal-6-sulfate [1] and leukocytes as the enzyme source. This originally reported assay relies on endogenous human β -galactosidase to release the 4MU after desulfation by GALNS. Extension of this assay to DBS involves supplementation of the cocktail with *Aspergillus oryzae* β -galactosidase (Sigma Chemicals) that is active at pH ~4 [2]. We attempted this GALNS assay with DBS and found that the commercial *Aspergillus oryzae* β -galactosidase from Sigma Chemicals was capable of generating 4MU from 4MU-Gal-6-sulfate (no difference in assay response was observed for assays with DBS versus filter paper, data not shown) suggesting that this preparation of enzyme is contaminated with a sulfatase. The calculation of the analytical range of this GALNS assay with DBS is not possible since the assay response for the blanks were not reported [2]. The authors reported a mean GALNS activity for DBS from healthy patients of 13.64 nmole per 48 h/mL of blood. This was measured with 10 mmol/l 4MU-Gal-6-S [2]. In the present study we report an activity of 3.8 μ mol per h/l blood (Figure 2) using 3 mmol/l 4MU-GalNac-6-S. This is 182 nmole per 48 h/ml of blood, which is 13-fold higher than the value reported with 4MU-Gal-6-S. We found that the GALNS activity increases linearly with the concentration of 4MU-GalNac-6-S in the 0–3 mmol/l range (not shown). This suggests that had the previous authors used 3 mmol/l 4MU-Gal-6-S, they would have measured ~3-fold lower activity, thus our best estimate is that 4MU-GalNac-6-S is used by GALNS ~40-fold faster than 4MU-Gal-6-S. From our mass spectrometry study we found that the GalNac-6-S is used about 48-fold faster by GALNS than the corresponding Gal-6-S substrate [4].

The new fluorescence assay for ARSB based on 4MU-GalNac-4-S shows separation between healthy and MPS-VI patients using DBS (Fig. 2). Good linearity is seen with the quality control DBS and as for GALNS, and the Y-intercept is not 0 suggesting residual ARSB in the base pool used to make the DBS standards (Figure 1). The analytical range for the high quality control sample is 6.3 (Table 1) versus 226 for the mass spectrometry assay [4].

The well-established assay for ARSB for diagnosis of MPS-VI uses the non-specific sulfatase substrate 4MU-sulfate (recent example, [3]) or related catechol sulfate substrates. Studies with DBS from MPS-VI patients show low but residual ARSB activity, suggesting that this substrate is detecting mainly ARSB. The source of this residual activity is not know. It may be due to residual activity of ARSB or due to one more additional human sulfatases that act on 4MU sulfate. Of interest is arylsulfatase A, the enzyme deficient in metachromatic leukodystrophy, since it hydrolyzes 4MU-sulfate [6]. Arylsulfatase A is active in samples using DBS since it can be detected after immunoprecipitation and subsequent assay with 4MU-sulfate [6]. Additional studies show that arylsulfatase A in DBS is relatively unstable [6]. All together the studies strongly suggest the use of the non-specific sulfatase substrate 4MU-sulfate is not appropriate for NBS of MPS-VI where fresh DBS will be the enzyme source. Caution is also warranted for diagnostic assays DBS and leukocyte samples that have not been confirmed to lack arylsulfatase A activity. This problem is

sometimes addressed by using different buffers to maximize either ARSB or arylsulfatase A activities, but complete separation of the two activities is not possible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations

ARSB	arylsulfatase B
DBS	dried blood spots on newborn screening cards GALNS, N-acetylgalactosamine-6-sulfatase
4MU	4-methyl-umbelliferyl
4MU-Gal-6-S	β -4MU glycoside of galactose-6-sulfate
4MU-GalNAc-4/6-S	beta-4MU glycoside of N-acetylgalactosamine-4/6-sulfate
MPS-IVA/VI	mucopolysaccharidosis-IVA/VI
NBS	newborn screening
β-NGA	β -N-acetylgalactosaminidase

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Highlights

- A new fluorimetric substrate for arylsulfatase B was developed.
- A new fluorimetric substrate for N-acetylgalactosamine-6-sulfatase was developed.
- Both substrates are based on the natural substrates.
- The new substrates can be used in fluorimetric assays of these enzymes.
- The assays are useful for the diagnosis of MPS types IVA and -VI.

Figure 1.

Activity versus the amount of DBS sample for GALNS (dots) and ARSB (triangles).

Activity values in Table 1 for the quality control blood spots are plotted versus the fraction of whole blood present in the base pool solution spotted on to DBS. The high sample (Table 1) is taken as 1, the medium as 0.5, and the low as 0.05.

Figure 2.
(left) GALNS activity in DBS from 46 healthy newborns and 2 MPS-IVA patients. (right)
For ARSB except with 51 healthy newborns and 3 MPS-VI patients.

Table 1

Performance parameters for fluorimetric assays of GALNS and ARSB.

Enzyme	Mean activity (%CV) $\mu\text{mol hr}^{-1} (\text{L blood})^{-1}$ for Quality Control DBS	Analytical Range Fluorescence	Analytical Range MS/MS [4]
GALNS	0.76 (10%) (Low) 5.2 (19%) (Medium) 7.87 (5%) (High)	5.0	198
ARSB	0.74 (35%) (Low) 3.09 (10%) (Medium) 4.98 (11%) (High)	6.5	226