HHS Public Access

Author manuscript

Mol Genet Metab. Author manuscript; available in PMC 2019 September 01.

Published in final edited form as:

Mol Genet Metab. 2018 September ; 125(1-2): 59–63. doi:10.1016/j.ymgme.2018.05.005.

Detection of Mucopolysaccharidosis III-A (Sanfilippo Syndrome-A) in Dried Blood Spots (DBS) by Tandem Mass Spectrometry

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Abstract

Background—With ongoing efforts to develop improved treatments for Sanfilippo Syndrome Type A (MPS-IIIA), a disease caused by the inability to degrade heparan sulfate in lysosomes, we sought to develop an enzymatic activity assay for the relevant enzyme, sulfamidase, that uses dried blood spots (DBS).

Methods—We designed and synthesized a new sulfamidase substrate that can be used to measure sulfamidase activity in DBS using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Results—Sulfamidase activity was readily detected in DBS using the new substrate and LC-MS/MS. Sulfamidase activity showed acceptable linearity proportional to the amount of enzyme and reaction time. Sulfamidase activity in 238 random newborns were well elevated compared to the range of activities measured in DBS from 8 patients previously confirmed to have MPS-IIIA.

Conclusions—This is the first report of an assay capable of detecting sulfamidase in DBS. The new assay could be useful in diagnosis and potentially for newborn screening of MPS-IIIA.

Keywords

lysosomal storage disease; enzyme deficiency; tandem mass spectrometry; dried blood spot; sulfamidase; mucopolysaccharidosis III type A

1. Introduction

Mucopolysaccharidosis III type A (MPS-IIIA) is an autosomal recessive inherited disease with no current treatment; however, intra-cerebrospinal fluid gene therapy and intrathecal enzyme replacement therapy have shown promising early clinical results [1,2]. MPS-IIIA patients have a deficiency in the activity of sulfamidase (SGSH, also known as heparan-N-sulfatase, EC 3.10.1.1), a lysosomal enzyme that removes sulfate from the nitrogen of glucosamine residues present in heparan sulfate. A fluorimetric assay of sulfamidase in fibroblasts has been reported using the 4-methylumbeliferyl-glycoside with glucosamine-N-sulfonate [3]; however, no assay of this enzyme in dried blood spots (DBS) suitable for newborn screening has been reported. In this study, we report the first assay of sulfamidase in DBS that is suitable for population screening and diagnosis of MPS-IIIA. The assay reported herein uses a novel substrate design in combination with tandem mass spectrometry (MS/MS) to measure the activity of sulfamidase in DBS.

2. Materials and Methods

2.1 Materials

All chemicals were reagent grade and were used as received. All patient samples were obtained with IRB approval. The methods described in this study are for research only and do not meet The Clinical Laboratory Improvement Amendments (CLIA) guidelines for the development of laboratory developed tests (LDTs). 2-Naphthol-1,3,4,5,6,7,8-d7 was purchased from Alfa Chemistry. Chemicals for synthesis were purchased from Sigma Aldrich. All experiments with human DBS were conducted in compliance with Institutional Review Board guidelines. All MPS-IIIA affected patients had been diagnosed previously with established clinical and biochemical procedures. Details of the syntheses of the substrates and internal standards are provided in the Supporting Information.

2.2 Standard Assay Procedure

Assay standard solution was made by mixing the MPS-IIIA substrate, 2-sulfamate-2-deoxy-1-α-(2-naphthyl)-glucopyranoside, and internal standard (IS), 2-amino-2-deoxy-1-α-(2-naphthyl-1,3,4,5,6,7,8-d_7)-glucopyranoside, in methanol to achieve a final concentration of 10 mM substrate and 10 μM IS. Assay buffer was 50 mM sodium acetate (reagent grade, J.T. Baker, Cat. 3460–01) and 1.59 mg/mL cerium (III) acetate hydrate (Sigma, 529559), pH 5.0 (adjusted on a pH meter calibrated with pH 4.0 and 7.0 standard buffers). Assay cocktail was prepared by removing the solvent of appropriate amount of assay standard solution with a centrifugal-concentrator or with a jet of oil-free air at room temperature. Then, assay buffer was added to give a final concentration of 1 mM substrate and 1 μM internal standard.

In a shallow 96-well plate (Greiner 651201), one 3 mm DBS was placed in each well and then 30 μL assay cocktail was transferred into each well. The plate was centrifuged in a swinging bucket rotor for ~1 min at ~2095 rcf to ensure that the DBS was fully immerged and all the liquid was at the bottom of the well. The plate was sealed with a silicone sealing matt and then placed on an orbital mixing platform (~250 rpm) at 37 °C for 16 hr. Enzymatic reactions were quenched by addition of 100 μL of acetonitrile, and the plate was centrifuged

for 5 min at room temperature at ~ 2095 rcf. A portion of the supernatant (90 μL) was transferred to an autosampler plate, and it was dried by a stream of nitrogen gas (or jet of oil-free air). The dried sample was then reconstituted with 90 μL of 90/10 H_2O/ACN. The plate was then wrapped with aluminum foil to minimize solvent evaporation and placed in the cooled (8 °C) autosampler chamber of the LC-MS/MS instrument.

2.3 LC-MS/MS

The column used in this study was ACQUITY CSH C18 UPLC, 1.7 μm, 2.1mm × 50 mm with a guard column (Waters Cat. 186005296 and 186005303). The column temperature was set at 40 °C. Solvent A was water/acetonitrile (90/10) with 0.1 % formic acid (all LC solvents are Optima grade from Fisher Scientific). Solvent B was acetonitrile/isopropanol (50/50) with 0.1% formic acid. The elution gradient in the LC was programmed as: 0–0.3 min (3% solvent B), 0.3–0.94 min (3% to 60% solvent B, linear gradient), 0.95–1.64 min (100% solvent B, step change at 0.95 min), 1.65–2.0 min (3% solvent B, step change at 1.65 min). The flow rate was 0.5 mL/min. For the autosampler, the weak needle wash was water/acetonitrile (90/10) with 0.1 % formic acid, and the strong needle wash was ACN with 0.1% formic acid.

MS/MS was carried out on a Waters Xevo-TQ instrument equipped with a Waters Acquity UPLC system. MS/MS instrument settings are given in Supplemental Material. Sulfamidase activity (μmole/h/L) was obtained by multiplying the ratio of ion counts of sulfamidase product to that of the internal standard by the μmole of internal standard added to the assay, then dividing by the incubation time (h) and volume of blood in one 3 mm DBS punch (3.2 uL).

3. Results and Discussions

3.1 Sulfamidase Substrates

In the two previous reports, the substrates for sulfamidase contained an N-sulfonated glucosamine monosaccharide attached to a non-saccharide aglycone [3,4]. These studies made use of leukocytes or skin fibroblasts, with no data reported for DBS. Our initial efforts to prepare a sulfamidase substrate that can be used with DBS was to use aglycones similar to the ones we reported earlier that show enhanced tendency to protonate in the gas phase for high sensitivity in MS/MS using electrospray ionization in positive-mode [5]. Figure 1A shows our designed sulfamidase substrate that contains the aglycone with a bis-amide functionality that is proposed to be an efficient site of proton capture [6]. However, when we incubated a 3 mm DBS punch with this substrate for 16 hours in a buffer previously shown to be optimal for sulfamidase[4], no enzymatic product could be detected by LC-MS/MS (data not shown). According to Freeman and coworkers [7], additional saccharides attached to the N-sulfonated-glucosamine can greatly enhance the activity with sulfamidase. We thus prepared α−1,4 disaccharide substrates containing either an iduronic acid moiety (Figure 1B) or a glucuronic acid moiety (Figure 1C) attached to the N-sulfonated-glucosamine. The synthesis of these substrates was based on previously developed chemistry for preparation of heparan sulfate fragments [4,8,9]. Unfortunately, these disaccharides failed to show detectable product by LC-MS/MS when incubated with a 3 mm DBS punch (data not

shown). For substrates shown in Figures 1A-C, we were able to detect the expected sulfamidase products when they were incubated with recombinant sulfamidase establishing that the products can be detected by LC-MS/MS (data not shown). We concluded that these substrates are not sufficiently active to detect the relatively small amounts of sulfamidase in DBS.

Another MPS III A substrate is commercially available for fluorimetric assay. It has a 4-methylumbelliferone (4-MU) as its aglycone. According to Karpova and coworkers [3], the 4-MU substrate for MPS III A has similar V_{max} as the one for a tetra-saccharide substrate [10], but the K_m of 4-MU substrate (4 mM) is significantly higher than that of tetra-saccharide substrates(10 – 30 μM). The previous report that used this 4-MU substrate for sulfamidase activity was for fibroblasts and leukocytes but not DBS. We tested the 4-MU substrate at 1 mM and 10 mM in the presence of a 3 mm DBS punch and buffer, but no product signal could be detected by LC-MS/MS (data shown in supplemental table 1). However, the product could be detected when recombinant sulfamidase was used.

The high V_{max} of the 4-MU substrate indicates that it is not necessary to have an extended saccharide moiety in sulfamidase substrate to achieve a large V_{max}. It can also be done by using an appropriate aglycone. Though the K_m will be higher, but a higher concentration of substrate is affordable since the synthesis of a monosaccharide substrate is vastly easier than the one for the polysaccharide substrate. By altering the aglycone structure, a novel compound containing N-sulfonated-glucosamine 1,4-linked to a 2-naphthyl aglycone (synthesis given in Supplemental Material) (named MPS-IIIA-S) was discovered (Figure 1D). This was the first substrate we synthesized that yielded detectable sulfamidase activity using a 3 mm DBS punch at 37 °C. A typical LC-MS/MS chromatogram is shown in Figure 2.

3.2 Optimization of sulfamidase activity with MPS-IIIA-S

We prepared and tested 9 analogs of MPS-IIIA-S containing structural modifications to the naphthyl aglycone. Results are summarized in Supplemental Table 1. Only one analog, with a 7-hydroxyquinoline aglycone, showed sulfamidase activity comparable to that of MPS-IIIA-S. Since the cost of synthesizing MPS-IIIA-S and its corresponding internal standard (IS) is much lower than its 7-hydroxyquinoline counterpart, we carried out all additional studies with MPS-IIIA-S.

Supplemental Figure 1 shows the activity of sulfamidase in DBS towards MPS-IIIA-S as a function of buffer pH. The pH profile was similar to the one reported by Hopwood and coworker [11]. Supplemental Figure 2 shows a hyperbolic dependence of the rate of sulfamidase activity versus the concentration of MPS-IIIA-S, and a K_M of 2.45 mM was determined from a fit of the data to the Lineweaver-Burk plot. All subsequent studies were carried out with 1 mM MPS-IIIA-S at pH 5.0. Product formation increases linearly as a function of incubation time (tested up to 40 hrs) (Supplemental Figure 3).

To test the inter-sample imprecision of the sulfamidase DBS assay, 8 different punches from the same DBS were assayed. The coefficient of variation (CV) is 2.7% obtained with the 8 activity values (0.106, 0.104, 0.107, 0.099, 0.108, 0.107, 0.104, and 0.108 μmol/L/h). This

indicates outstanding reproducibility of the new sulfamidase assay and well within the range seen for other NBS assays.

We tested the sulfamidase assay with quality control DBS from the Centers for Disease Control and Prevention (CDC) (QC Low, Medium, and High), which were prepared by mixing leukocyte-depleted and heat treated blood with various amounts of whole blood [12]. As shown in Figure 3, a linear relationship was observed for the sulfamidase activity versus the fraction of whole blood in the QC standards. When a DBS from a healthy adult was used in the assay, the sulfamidase activity was 0.176 μmol/L/h compared to 0.008 μmol/L/h when a filter paper punch (no blood) was submitted to the same assay protocol (Figure 4). This 22-fold difference is lower than the blood-to-no blood ratios seen with other MS/MS assays of lysosomal enzymes [13] in DBS but still adequate for screening as it is larger than values typically seen with fluorimetric assays of the same enzymes [14].

Temperature-dependent studies of sulfamidase activity are summarized in Supplemental Figure 4. When the temperature was increased from 37 °C to 60 °C, sulfamidase activity increased ~5-fold, and the filter paper blank remained acceptably low at all temperatures. Most NBS laboratories will presumably not choose to carry out the MPS-IIIA assay at the elevated temperatures, but this is an option for diagnostic reference labs in cases where an expansion of the range of enzymatic activities may be desirable.

Sulfamidase activity shows excellent stability in DBS as shown in Supplemental Figure 5. Sulfamidase activities were around 0.25 μmol/L/h for DBS stored at both −20 °C and 4 °C over the course of 68 days. When stored at room temperature, sulfamidase activity decreased about 50% over the same period. Thus, loss of activity of sulfamidase during sample shipment should be minimal.

3.3 Sulfamidase assays with normal and MPS-IIIA-deficient samples

As shown in Figure 4, sulfamidase activity was readily detected in a DBS made using blood from a wild-type mouse (9.77 μmol/L/h) but was virtually absent using a DBS from a sulfamidase knockout mouse (0.013 μmol/L/h). Note that the wild-type mouse has 55-fold higher sulfamidase activity compared to a healthy adult, but this activity is virtually all due to a single gene product based on the data with the knockout.

The sulfamidase assay was carried out with 3 mm DBS punches from 238 random newborns, and from 8 patients clinically diagnosed with MPS-IIIA, and results are shown in Figure 5. Seven of 8 MPS-IIIA patients had sulfamidase activity of less than 0.02 μmol/L/h, and one patient had an activity of 0.056 μmol/L/h. The random newborns had activity in the range 0.10 – 0.81 μmol/L/h with a mean activity of 0.3 μmol/L/h. Thus, all MPS-IIIA patients separate from the large cohort of non-MPS-IIIA newborns.

Figure 6 shows that the sulfamidase activities for the 238 random newborn DBS fit well to a log-normal distribution. The method for the fitting is described in the Supplemental Material. Based on the fit curve, if a cutoff value for sulfamidase was set at 20% of the mean activity of newborns (above the range of activities seen with DBS from 8 MPS-IIIA patients) the false positive rate would be 1.4×10^{-3} %.

4. Conclusions

In this study, we report the first assay of sulfamidase in DBS for evaluation of MPS-IIIA. All other previously reported sulfamidase assays made use of blood leukocytes or skin fibroblasts. The assay reported herein is appropriate for high-throughput NBS laboratories and reference laboratories.

The novel substrate exhibits higher V_{max} and K_m in a DBS assay for sulfamidase, which agrees with Karpova's [3] finding. The extension of saccharide unit in the substrate for MPS-IIIA lowers its K_m significantly but it does not necessarily correlate to a higher V_{max}. The reason behind this finding is unknown. With the considerably lower cost of synthesizing the 2-naphthol substrate compared to the polysaccharide substrate, a higher substrate concentration in the actual assay is still affordable.

Since the new sulfamidase assay is carried out with LC-MS/MS, it will be possible to add it to our other LC-MS/MS assays for LSDs [13,15] to continue to expand the multiplex capabilities of this platform for NBS and diagnosis of lysosomal storage diseases. Such expansion could be considered if an approved therapy for MPS-IIIA is developed. We plan to initiate pilot studies using the new sulfamidase assay in a NBS lab to explore the robustness of the assay and the number of false positives.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was funded by a grant from the National Institutes of Health to M. H. Gelb, F. Turecek, and C. Ronald Scott (R01 DK067859).

Abbreviations

LC-MS/MS	liquid chromatography-tandem mass spectrometry
MPS-IIIA	mucopolysaccharidosis III type A
DBS	dried blood spot
SGSH	sulfamidase

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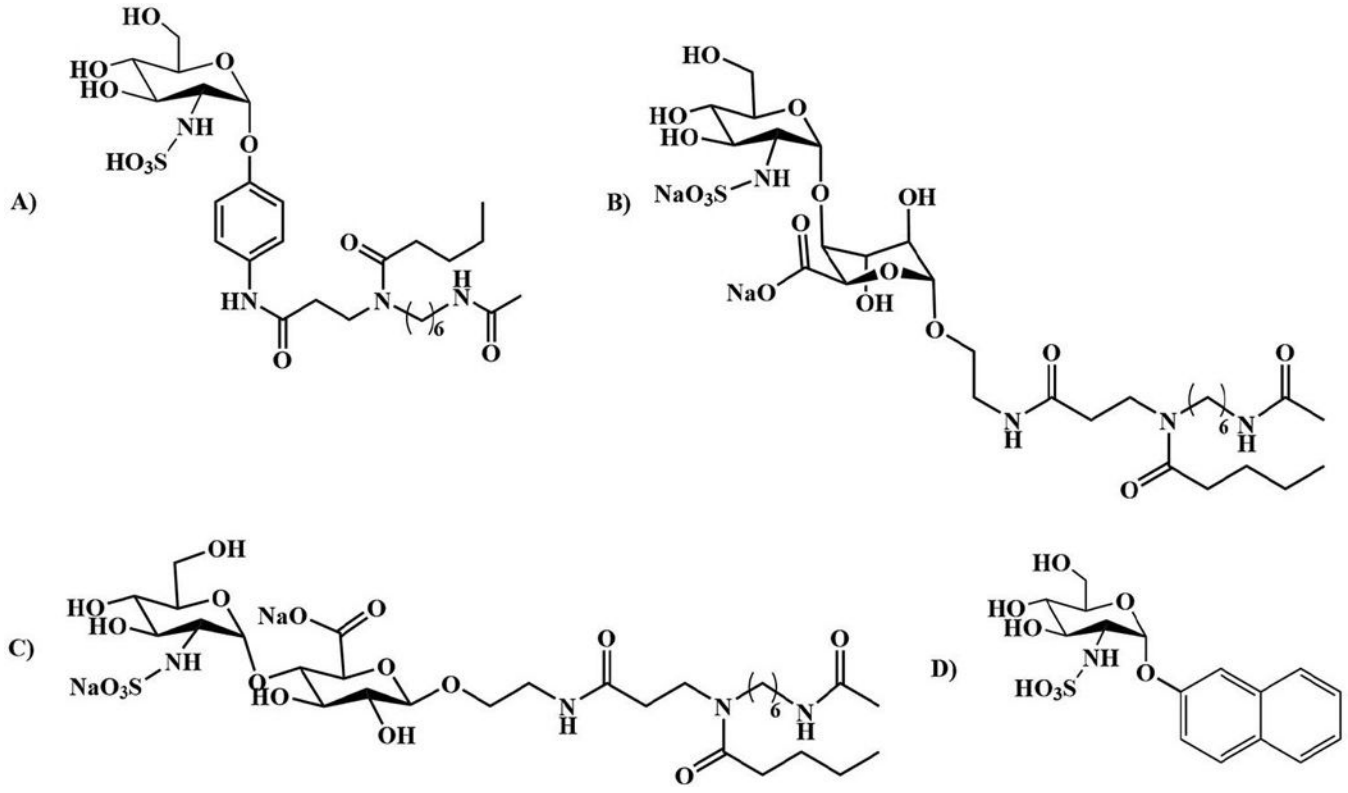

Figure 1.

Structures of sulfamidase substrates. A) Our original design with a monosaccharide moiety and an aglycone that readily protonates in the gas phase during MS/MS; B) Disaccharide substrate with iduronic acid linked to the N-sulfate-glucosamine. C) Disaccharide substrate with glucuronic acid linked to the N-sulfate-glucosamine. D) MPS-IIIA-S.

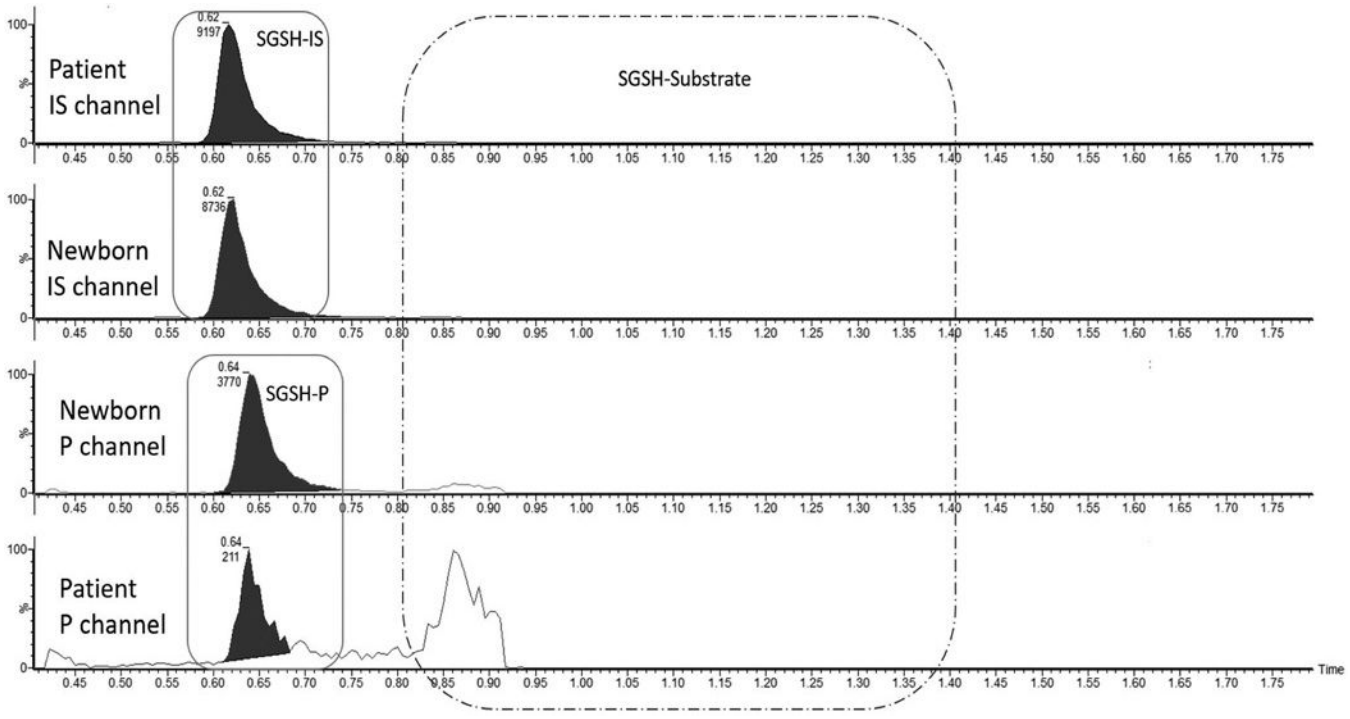

Figure 2.

Typical LC-MS/MS chromatograms for Sulfamidase assay with DBS. Top two panels is the internal standard channel for MPS-IIIA patient and healthy newborn, respectively, and bottom two panels is the MPS-IIIA product channel. MPS-IIIA-S elutes as a broad peak around 1.22 min (starting to elute at 0.86 min) and gives rise to the observed product ions due to cleavage of MPS-IIIA-S in the heated electrospray ionization source. Since this peak elutes well after the product due to the action of sulfamidase, this in-source product peak is of no concern. At ~0.9 min, the eluent from the LC column is diverted to waste rather than to the MS/MS via a switching valve. MPS-IIIA-S continues to elute from the column out to ~1.4 min.

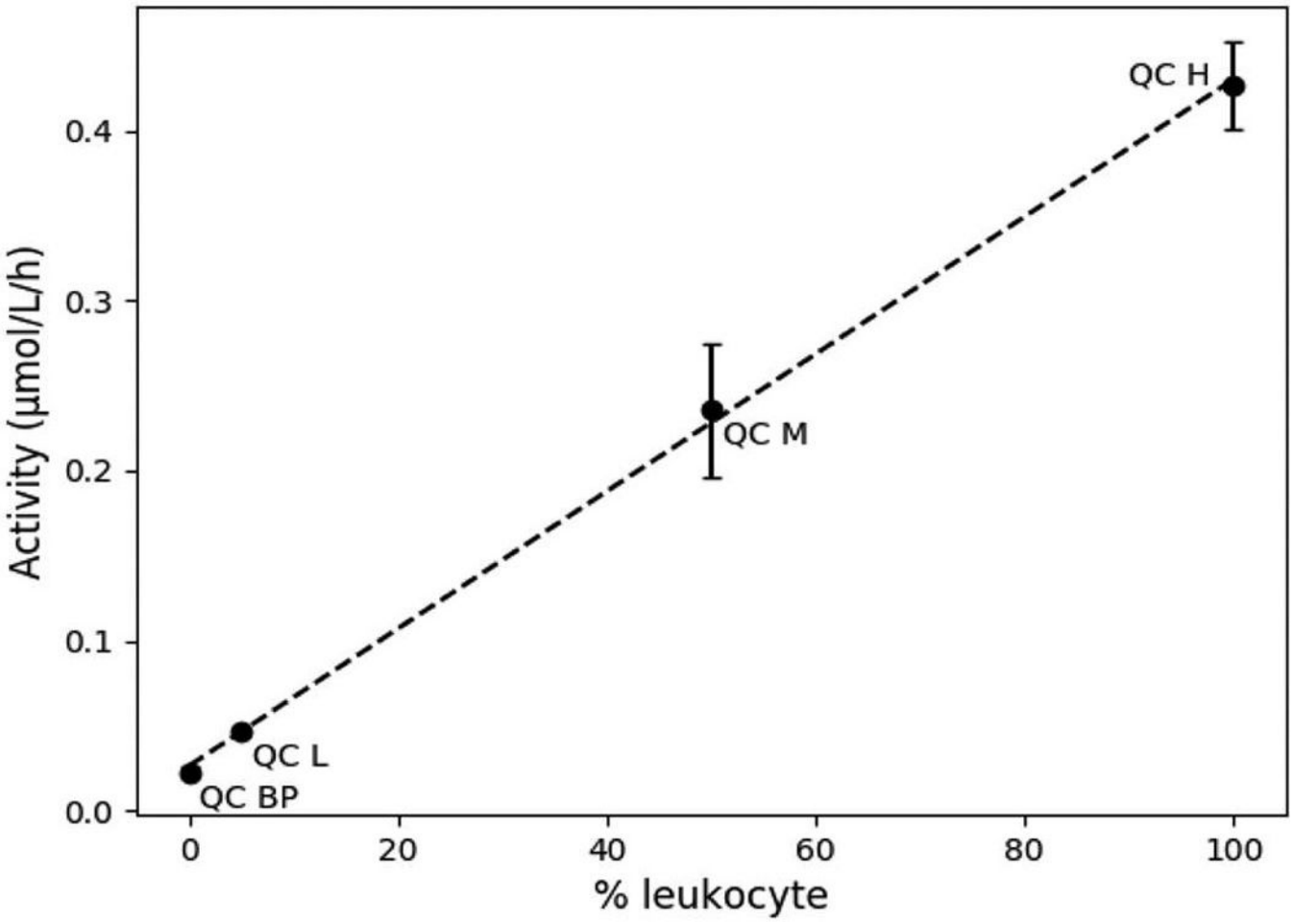

Figure 3.

Sulfamidase assay with CDC quality control DBS samples. QC BP: quality control base pool with 0% normal leukocyte; QC L: quality control low with 5% normal leukocyte; QC M: quality control medium with 50% normal leukocyte; QC H: quality control high with 100% normal leukocyte.

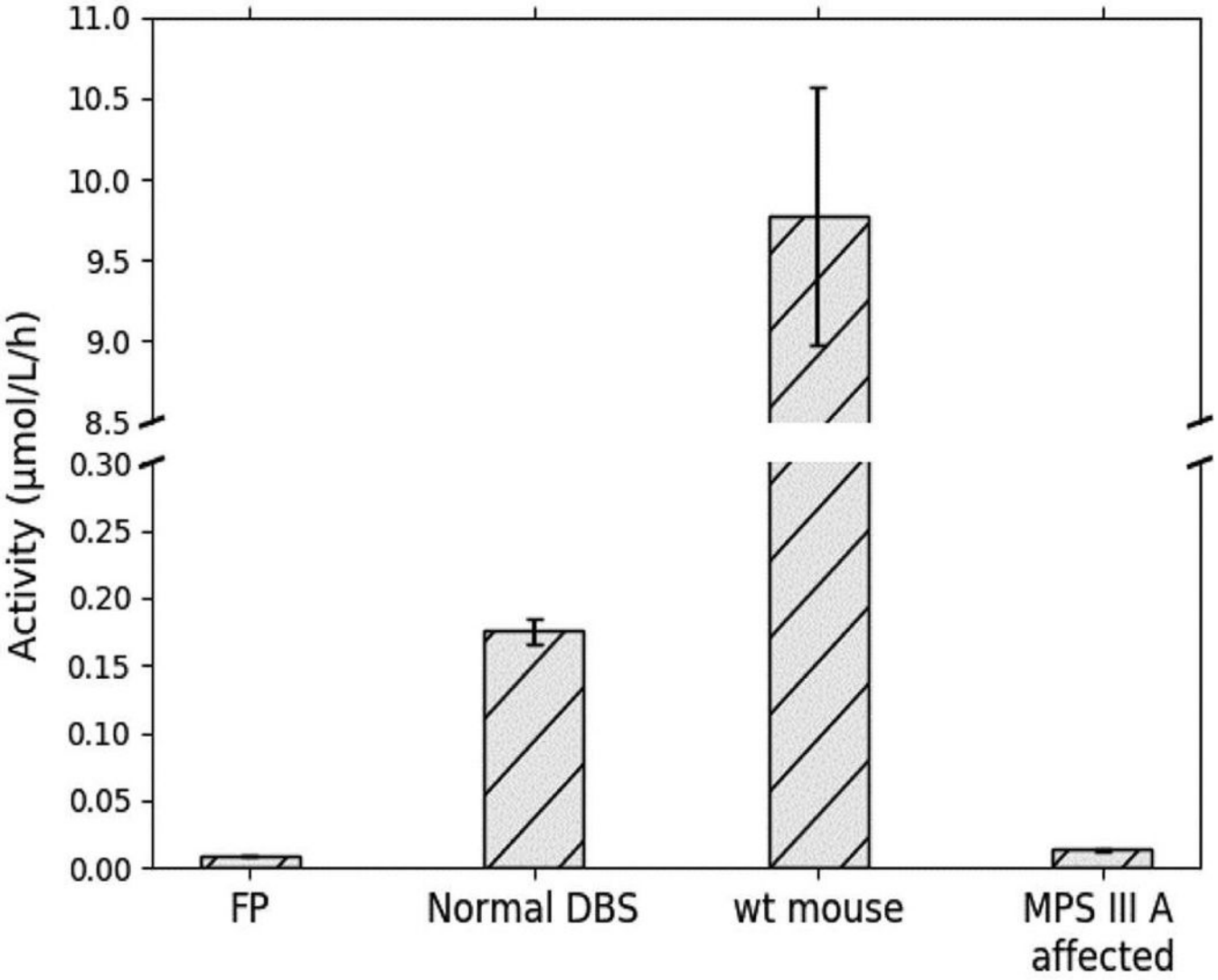

Figure 4.
MPS-IIIA assay with a DBS from a healthy human (0.008 µmol/L/h), DBS from a wild-type mouse (9.77 µmol/L/h), and DBS from a sulfamidase-knockout (null) mouse (0.013 µmol/L/h).

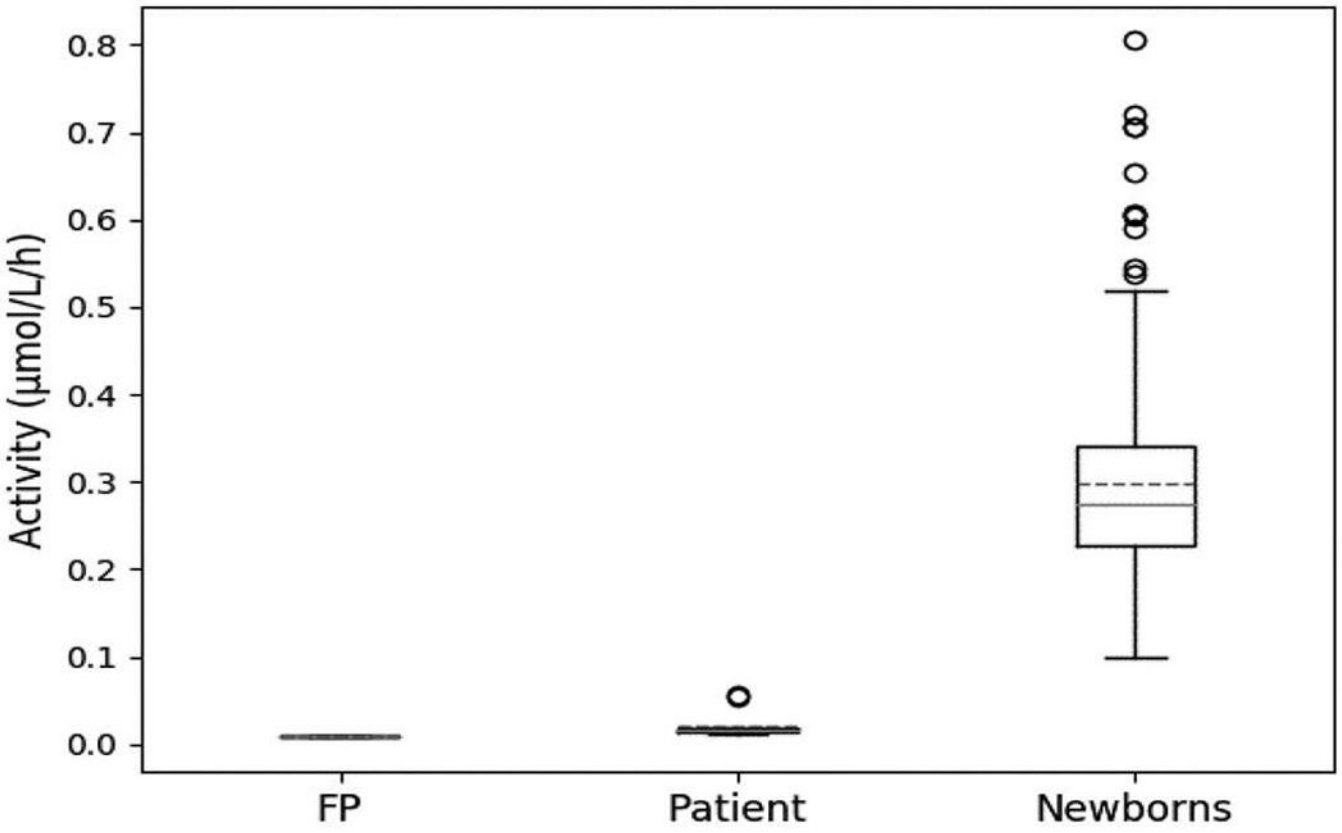

Figure 5.

MPS-IIIA assay with filter paper (no blood DBS, 0.008 µmol/L/h), DBS from 8 MPS-IIIA patients (0.013–0.056 µmol/L/h), and DBS from 238 random newborns (0.10–0.81µmol/L/h).

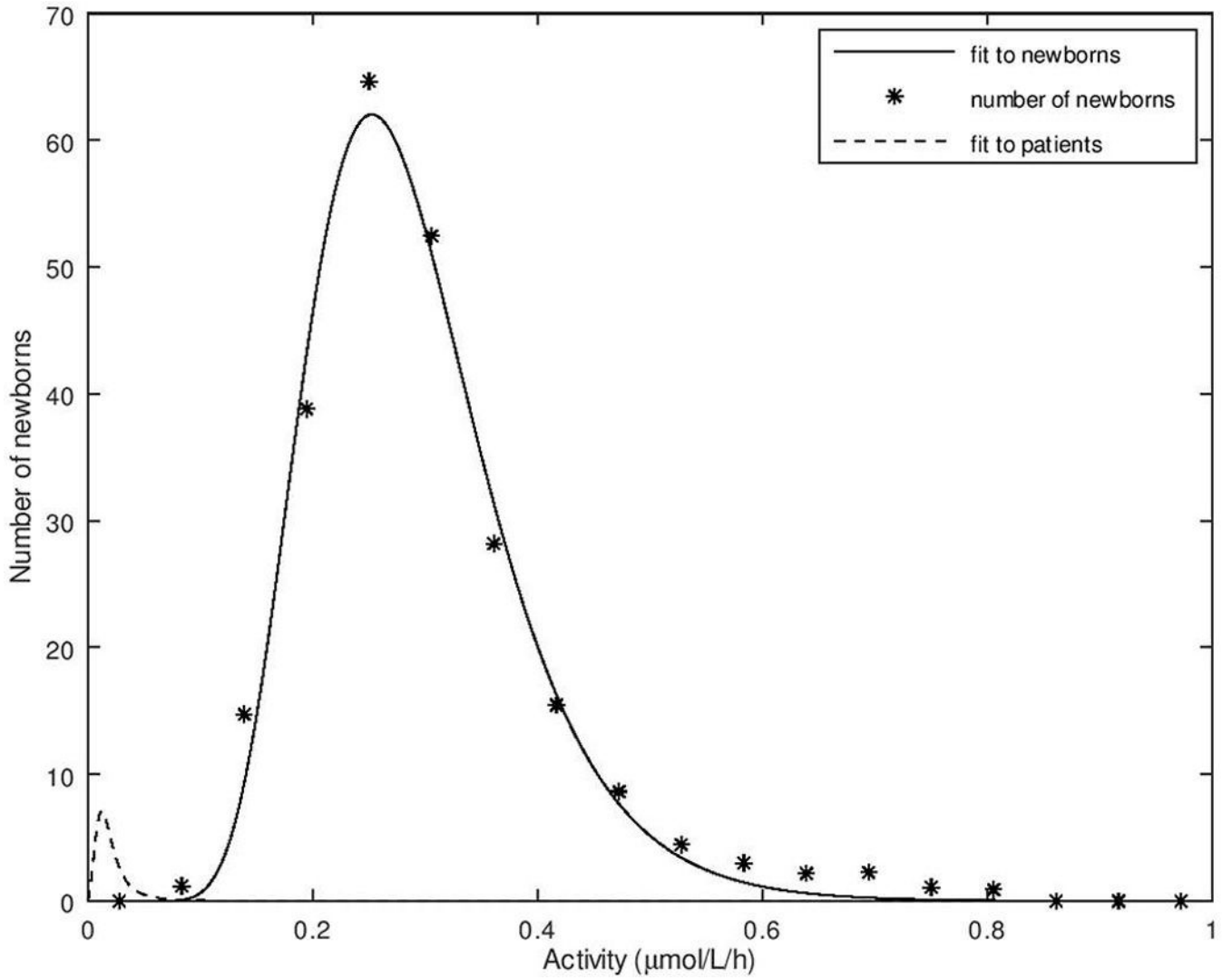

Figure 6.

A log-normal-distribution fit of the newborns and MPS-IIIA patient sulfamidase activity. The solid black line represents the fit to newborns and the dotted line represents the fit to patients. Details of the fitting method are given in Supplemental Material.