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Tandem Mass Spectrometry for the Direct Assay of Lysosomal Enzymes in Dried Blood Spots: Application to Screening Newborns for Mucopolysaccharidosis VI (Maroteaux-Lamy Syndrome)

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We report a new assay of *N*-acetylgalactosamine-4-sulfatase (aryl sulfatase B) activity in dried blood spots (DBS) for the early detection of mucopolysaccharidosis VI (Maroteaux−Lamy syndrome) in newborn screening. The assay uses a synthetic substrate consisting of *N*-acetylgalactosamine-4-sulfate moiety glycosidically linked to a hydrophobic residue and furnished with a *tert*-butyloxycarbamido group as a marker for specific mass spectrometric fragmentation. Incubation with aryl sulfatase B present in DBS converts the substrate to a desulfated product which is detected by electrospray tandem mass spectrometry and quantified using a homologous internal standard. Assay and workup procedures were optimized to be compatible with the work flow in newborn screening laboratories. Analysis of DBS from human newborns showed clear distinction of aryl sulfatase B activity from 89 healthy individuals where it ranged between 1.4 and 16.9 μmol/(h L of blood), with an average activity of 7.4 μmol/(h L of blood), and an MPS-VI patient that had an activity of 0.12 μmol/(h L of blood). Results are also reported for the aryl sulfatase B assay in DBS from groups of normal felines and felines affected with MPS-VI.

The mucopolysaccharidoses (MPS-I to VII) are a group of metabolic diseases caused by a deficiency of one of the lysosomal enzymes degrading the glycosaminoglycans (GAG) heparan, dermatan, keratan, or chondroitin sulfate.[1] The pertinent enzymes consist of five sulfatases, four exoglycosidases, and one nonhydrolytic acetyl-*N*-transferase. Their clinical features exhibit multisystem involvement with a chronic and progressive course, although the individual phenotypes vary depending on the disease and its severity. Mucopolysaccharidosis VI (Maroteaux−Lamy syndrome)[2,3] is caused by deficiency of *N*-acetylgalactosamine-4-sulfatase (also called aryl sulfatase B, ASB, EC 3.1.56.12)[4−6] which is one of several enzymes involved in stepwise degradation of chondroitin and dermatan sulfate (Scheme 1). ASB deficiency is inherited as an autosomal recessive trait and causes an accumulation of partially degraded material in the lysosome resulting in organ damage. The affected children may appear normal at birth but within 1−3 years develop skeletal abnormalities causing short stature and joint stiffness as well as other symptoms such as corneal clouding. Treatment of MPS-VI by enzyme replacement therapy (ERT)[7,8] has been developed by BioMarin Pharmaceuticals Inc. (Naglazyme) and approved for clinical use since 2005. Thus, there is a need for developing a fast, inexpensive, and reliable diagnostic procedure that uses the dried blood spots (DBS) submitted to newborn screening laboratories.

Previous assays that have been developed to diagnose MPS-VI used 4-methylumbelliferone sulfate as a fluorogenic enzyme substrate. It has been demonstrated that ASB retains latent activity in dried blood spots.[9,10] However, this phenolic sulfate is a substrate for many of the enzymes in the sulfatase family, so the fluoroscence-based assay lacks selectivity. A radiometric assay has

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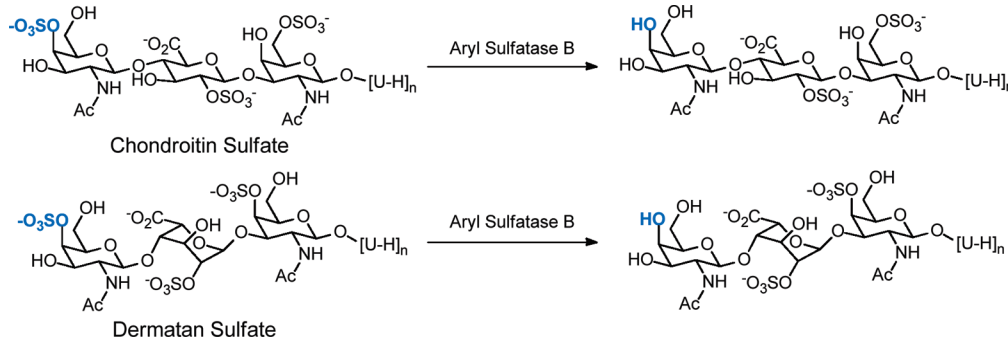
Scheme 1



been reported that uses a labeled trisaccharide that is enzyme specific but difficult to synthesize on a large scale.[11]

We have been developing specific assays for lysosomal enzymes in dried blood spots[12−14] that have been or are in the process of being applied in newborn screening laboratories in the U.S.[15−17] and abroad.[18,19] Our approach relies on rehydrating the enzyme in a dried blood spot in an appropriate buffer and assaying it by incubation with a synthetic substrate. The substrates are designed to closely mimic the structure motif acted upon by the enzyme in the natural GAG and have been shown to be highly specific, exhibiting no cross activity among sulfatases.[20] The enzyme activity is determined by measuring the enzymatic product relative to an added internal standard by electrospray ionization tandem mass spectrometry (ESI-MS/MS). ESI-MS/MS assays for several lysosomal storage disorders, including glycosaminoglycan-related MPS-I (Hurler syndrome),[21] MPS-II (Hunter syndrome),[22] and MPS-IV (Morquio A syndrome),[23] have been developed to be applied in newborn screening. Here we report a new MS/MS assay of aryl sulfatase B for the detection of MPS-VI. The goal was to develop a specific assay to diagnose MPS-VI that could be used in a multiplex fashion for the determination of several sulfatases where enzymatic products and internal standards indicative of other lysosomal enzyme activities are simultaneously sampled and analyzed by MS/MS.

EXPERIMENTAL SECTION

Materials. The substrate (7-*O*-(2-acetamido-2-deoxy-4-*O*-sulfonato-β-D-galactopyranosyl) *tert*-butyl 6-(7-hydroxy-coumarin-4-acetamido)hexylcarbamate sodium salt, ASB-S) and internal standard (7-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl) *tert*-butyl 5-(7-hydroxy-coumarin-4-acetamido)pentylcarbamate, ASB-IS) were synthesized in several steps, and their purity was checked by ^{1}H NMR and electrospray mass spectra, as reported elsewhere.[24] Dried blood spots (DBS) from anonymous newborns were obtained from the Washington State Newborn Screening Laboratory with approval of the Washington State Institutional Review Board (IRB). Additional DBS from two unaffected adult donors were used to develop the assays and to characterize the enzyme kinetics. DBS from a single affected anonymous donor were obtained from BioMarin Pharmaceutical Inc. (Novato, CA). The MPS-VI affected patient had been diagnosed previously with established clinical and biochemical procedures. DBS from normal and affected felines were obtained from BioMarin Pharmaceutical Inc. 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 $CaSO_4$ granules).

Standard Assay Using Liquid−Liquid Extraction. A 3 mm punch from a dried blood spot was placed in a single well of a 96-deep well plate (1 mL, Costar, Fisher Scientific, catalog no. 09-761-116) containing 20 μL of assay cocktail (100 mM sodium formate, pH 4.0, 30 mM lead(II) acetate, 1 mM substrate (ASB-S), and 5 μM internal standard (ASB-IS)). The plate was centrifuged briefly to bring all components to the well bottom. The plate was sealed and incubated at 37 °C for 16 h in a thermostatted air shaker. The sample was quenched with 100 μL of water containing 16 mg of diethylaminoethylcellulose resin (DEAE cellulose, 20 mg, Whatman, catalog no. 4057-200), leading to a precipitate. A blank assay was carried out as above but using a blank paper punch instead of a DBS. Samples were submitted to liquid−liquid extraction with the addition of 400 μL of ethyl acetate. The solutions were mixed by aspiration with a 12-channel pipet (10×) and then centrifuged at 3000 rpm for 5 min to separate the layers. A volume of 300 μL of the top layer was transferred to a new 96-

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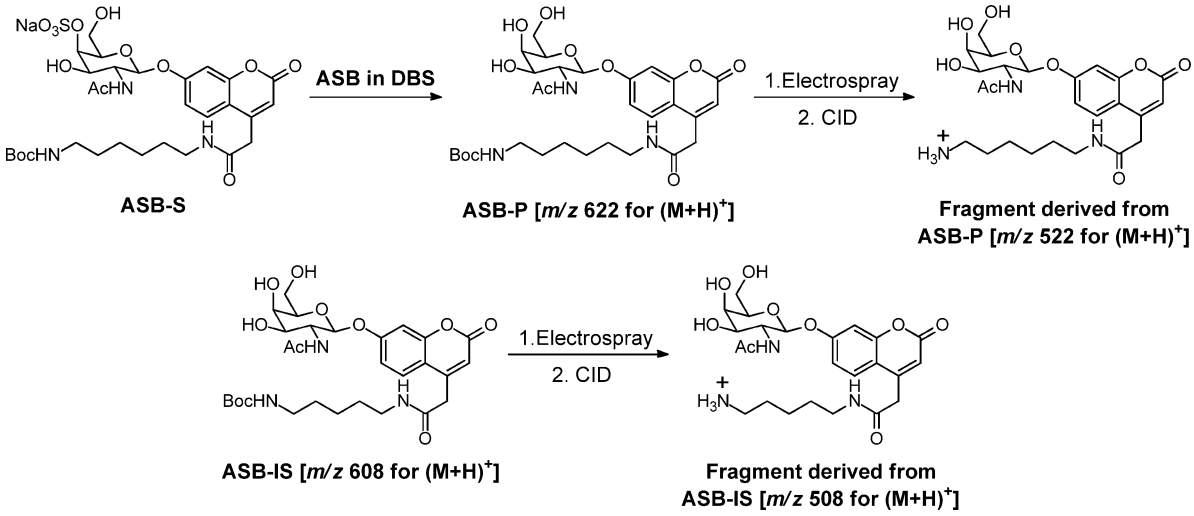
Scheme 2



well plate (0.5 mL, Axygen Scientific, VWR International, catalog no. 47743-982). The ethyl acetate was removed under a stream of air, and the sample was reconstituted in 100 μL of 80/20 acetonitrile/water with 0.2% formic acid.

Standard Assay Using Solid Phase Extraction. A 2 mm punch from a dried blood spot was placed in a 0.5 mL polypropylene tube (Eppendorf) containing 20 μL of assay cocktail (100 mM sodium formate, pH 4.0, 30 mM lead(II) acetate, 1 mM substrate (ASB-S), and 5 μM internal standard (ASB-IS). The solution was vortexed briefly then centrifuged briefly to bring all components to the tube bottom. The capped tube was incubated at 37 °C for 16 h in a thermostatted air shaker. The sample was quenched with 100 μL of 25 mM Na_2HPO_4, leading to a precipitate. A blank assay was carried out as above but using a blank paper punch. Samples were submitted to solid phase extraction using a vacuum manifold (Millipore Inc., MAVM0960R) system connected to an aspirator. DEAE cellulose resin (20 mg) in acetic acid (250 μL) was pipeted into each well of a 96 well filter plate (Innovative microplate, catalog no. F20005). The ion-exchange resin was washed with methanol (2 × 0.5 mL). A slurry of C-18 resin (20 mg, Aldrich, octadecyl-functionalized silica gel, catalog no. 377635) in methanol (250 μL) was pipeted on top of the ion-exchange resin in each well. The 2 resin layers were washed with methanol (2 × 0.5 mL) to help it settle, then with deionized water (2 × 0.5 mL). The sample was slowly loaded onto the column and washed with deionized water (2 × 0.5 mL) to remove salts. Finally the product and internal standard were eluted with methanol (2 × 0.5 mL) into a deep well plate (Neptune, catalog no. 2405). The methanol was removed in a centrifugal concentrator (Speed-Vac), and the sample was reconstituted in 30 μL of 80/20 acetonitrile/water with 0.2% formic acid.

Mass Spectrometry. ESI-MS/MS analysis was performed on a Waters Quattro Micro tandem quadrupole instrument using flow injection and selected reaction monitoring in the positive ion mode. Samples were injected manually using a syringe, except for the samples using the liquid−liquid extraction protocol that were injected using an autosampler. In total, 25 μL of the 100 μL sample was injected using a Waters 2777C Sample Manager via flow injection of 80/20 acetonitrile/water with 0.2% formic acid with a flow rate of 0.1 mL/min for 1 min then 1 mL/min for 0.5 min. Data was collected during 1.5 min of infusion, and the signal returned to the background level before the next injection. The ion dissociations used for selected reaction monitoring were m/z 608.3 → m/z 508.6 and m/z 622.3 → m/z 522.6 for the internal standard and product, respectively. Other ESI-MS/MS conditions were as follows: electrospray capillary voltage, 4500 V; extractor, 3 V; desolvation temperature, 350 °C; desolvation gas flow, 600 L/h; collision cell pressure, 2.23×10^{-3} millibar. The cone voltage and ion laboratory collision energy were 15 V and 13 eV, respectively, for both m/z 622.3 → m/z 522.6 and m/z 608.3 → m/z 508.6 transitions. The dwell time was 100 ms with a 20 ms delay. The amount of ASB product was then determined from the ion intensity ratio of the product to internal standard and converted to ASB activity (μmol/(h L of blood)) using the incubation time and blood volume in the DBS punch. The mean volume of blood was estimated to be 10 μL per DBS. The amount of blood in the punch was calculated as 3.2 μL from the punch/DBS area ratio.

RESULTS AND DISCUSSION

The strategy for the MPS-VI assay using tandem mass spectrometry is outlined in Scheme 2. The enzyme in DBS is incubated with ASB-S to catalyze specific hydrolysis in the *N*-acetylgalactosamine-4-*O*-sulfate moiety yielding ASB-P. The product is ionized by protonation in electrospray to produce the m/z 622 precursor ion, $(M + H)^+$, which is selected by mass and subjected to collision induced dissociation (CID) forming the m/z 522 product ion. CID is steered into one predominant product ion channel by the *tert*-butyloxycarbamido (*t*-BOC) group that undergoes facile elimination of isobutene and carbon dioxide.[14] The internal standard (ABS-IS) is a lower homologue of ASB-P from which it differs in the length of the diamine carbon chain which has six methylene groups in ASB-P and five methylene groups in ASB-IS. CID of the ASB-IS $(M + H)^+$ ion forms an m/z 508 fragment ion which is homologous with the m/z 522 fragment from ASB-P. The ASB-P and ASB-IS structures were designed such that the m/z values of both precursor and fragment ions were distinct from those of substrates and internal standards used in ESI-MS/MS assays

of other lysosomal enzymes. Thus, the ASB assay can be multiplexed with any other previously developed ESI-MS/MS assays.

The relative response in ESI-MS/MS to ASB-P and ASB-IS concentrations in the sample, R_P/R_{IS}, depends on the partition coefficients in extraction from the assay buffer, electrospray ionization efficiency, and propensity for fragmentation by elimination of isobutene and CO_2. The R_P/R_{IS} ratio was established as 2.09 from a calibration curve which showed excellent linearity ($r^2 = 0.994$) for ASB-P/ASB-IS concentration ratios ranging from 0.1 to 5.0 (Figure S1, Supporting Information). This range of ASB-P concentrations corresponds to 10−500 pmol of enzymatic product formed in an assay, which is consistent with the range of enzyme activities found in DBS (vide infra).

The assay conditions and work up procedures were thoroughly investigated and optimized. Assays of lysosomal enzymes are typically conducted at low pH 3.5−5.3 and under conditions achieving low substrate conversions (<10%) to keep the enzyme kinetics in the initial pseudolinear stage and also to avoid enzyme inhibition by the products. Sulfatases, in particular, are inhibited by free sulfate ions present in the sample or produced by enzymatic hydrolysis.[25] Inorganic sulfate is sequestered as insoluble lead(II)sulfate ($pK_{sp} = 6.20$) by in situ precipitation with soluble lead salts, such as lead formate or acetate. The effect of lead formate on the ASB activity is shown in Figure S2 in the Supporting Information. In the absence of a lead salt, ASB showed no activity toward ASB-S but full activity was recovered at lead concentrations >20 mM. Lead formate and acetate showed the same effect, and so 30 mM lead(II) acetate was used in all ASB assays.

One of the advantages of MS/MS-based assays is the capacity for multiplexing analysis of several enzyme reactions in a single injection into the mass spectrometer.[26] For practical purposes of work time constraints and sample throughput in newborn screening laboratories, it is also desirable to carry out the incubation of several enzymes in one common buffer.[27] Hence, effects of pH on the enzyme activity need to be studied to find a pH range where several sulfatases might have sufficient activity. The ASB activity showed a 3-fold increase from pH 3.4 to pH 4.0 (Figure S3 in the Supporting Information) and then the dependence flattened at pH 4−4.5. The other parameters of the ASB assay were studied under the optimized conditions of pH 4 and 30 mM lead concentration. Figure S4 in the Supporting Information shows that the amount of ASB-generated product in DBS increased as a linear function of incubation time between 5 and 30 h. This indicated that the assay conditions were such that the enzyme kinetics was in the initial stage. The incubation time was set to 16 h, which is compatible with the work schedule in newborn screening laboratories.

Michaelis−Menten parameters were measured as shown in Figure S5 in the Supporting Information. The amount of product formed increased in a hyperbolic fashion versus substrate concentration measured between 0.1 and 1 mM to give $K_M = 0.45$ mM after a nonlinear regression fit. A substrate concentration of 1 mM was used to saturate the enzyme in an effort to minimize the effect of potential competitive inhibitors present in blood. The amount of product formed increased with the size of the DBS punch (Figure S6 in the Supporting Information), with a plateau at higher blood amounts, presumably due to an increase in endogenous inhibitors. A 3 mm DBS punch was used to be compatible with the protocols used in newborn screening laboratories.

Table 1. ASB Activities for Individual DBS in Humans Using Solid Phase Extraction Method

sample	enzyme activity in μmol/(h L of blood)
blank[a] ($n = 3$)	0.00
MPS-VI patient	0.11
newborns 1−10	2.2, 12.9, 4.6, 4.5, 14.5, 11.3, 3.8, 3.8, 3.3, 10.5

[a] Blanks used a blank paper punch instead of a blood spot.

Postincubation purification was necessary due to the high concentration of buffer salts, which would interfere with electrospray ionization. In addition, we found that the sulfated substrate (ASB-S) undergoes fragmentation upon electrospray and ion transfer to the vacuum system that resulted in dissociative desulfation forming ABS-P ions. Although this dissociation is a minor process, it gains importance due to the large excess of ABS-S in the assay sample and produces a large background signal in MS/MS. Both the buffer salts and the sulfated substrate could be easily removed with a single liquid−liquid extraction step using ethyl acetate and water containing an anion exchange resin. The inorganic buffer salts partitioned into the aqueous layer and the anionic sulfate substrate was retained on the anion exchange resin.

Two procedures were developed to isolate the hydrophobic ASB-P and ASB-IS. Early investigation of the assay utilized a solid-phase extraction procedure using C18 silica gel and DEAE cellulose resin in an acetate form. The results obtained with solid phase extraction are listed in Table 1, and the distribution of activities is shown by the blue bars in Figure 1. The measurements showed negligibly low blanks and a 2.2−12.9 μmol/(h L of blood) range of enzyme activities for healthy newborns. The affected patient showed a substantially lower activity (0.11 μmol/(h L blood) that was clearly separated from those of the healthy individuals.

The sample workup using solid-phase extraction requires multiple liquid transfers which may be cumbersome in a newborn screening laboratory setup. Therefore, we developed an alternative procedure using liquid−liquid extraction to ethyl acetate. This procedure is more expedient as it involves fewer liquid transfer steps and thus facilitates a high-throughput execution of the assay. With the use of the optimized assay conditions and the liquid−liquid extraction protocol, ASB activities in DBS from 89 unaffected newborns and 1 MPS-VI patient were analyzed. The unaffected newborns displayed an activity range of 1.4−16.9 μmol/(h L of blood), with an average activity of 7.4 μmol/(h L of blood) (Table 2). The distribution of activities is shown as black bars in Figure 1. The DBS from a previously identified MPS-VI patient gave a value of 0.12 μmol/(h L blood), which was very close to the activity measured with the method using solid phase extraction (Table 1). Quality control DBS provided by the Centers for Disease Control and Prevention were also analyzed for ASB activity. These

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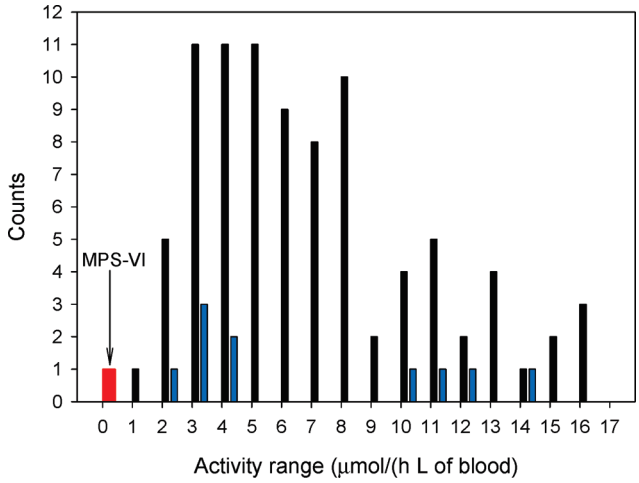

Figure 1. Distribution of ASB activities in DBS from humans. Black bars show data for 89 unaffected newborns obtained using the liquid−liquid extraction method. Blue bars show data for 10 unaffected newborns obtained using the solid phase extraction method. The red bar is for 1 MPS VI patient. ASB activity values for each sample are given in Tables 1 and 2.

Table 2. ASB Activities for Individual DBS in Humans Using Liquid−Liquid Extraction Method

sample	enzyme activity in μmol/(h L of blood)
blank	0.17 ± 0.08
CDC QC base	0.20 ± 0.20 (n = 3)
CDC QC low	0.65 ± 0.26 (n = 3)
CDC QC medium	3.4 ± 0.35 (n = 3)
CDC QC high	8.8 ± 2.0 (n = 3)
MPS-VI patient	0.12
random newborns 1−12	6.2, 7.1, 7.6, 16.9, 16.2, 3.2, 2.8, 5.6, 10.7, 3.9, 15.5, 4.8,
random newborns 13−24	3.6, 9.8, 13.8, 11.0, 5.2, 4.2, 3.3, 11.7, 8.7, 5.8, 8.7, 4.9,
random newborns 25−36	7.7, 3.3, 5.5, 10.0, 6.2, 2.1, 5.6, 13.2, 8.5, 6.8, 2.7, 4.2,
random newborns 37−48	3.6, 4.6, 7.2, 4.5, 6.1, 5.4, 2.0, 8.1, 8.6, 9.9, 8.8, 5.3,
random newborns 49−60	1.4, 8.3, 8.1, 13.0, 8.5, 6.5, 6.1, 11.8, 12.7, 13.1, 5.7, 15.0,
random newborns 61−72	3.9, 15.8, 11.9, 6.6, 6.8, 11.2, 3.0, 12.4, 2.8, 8.7, 6.9, 7.6,
random newborns 73−84	5.5, 4.3, 4.3, 10.5, 7.6, 7.3, 3.1, 16.5, 4.7, 3.7, 10.7, 4.1,
random newborns 85−89	3.6, 4.2, 5.4, 5.8, 7.9

samples are prepared from fully and partially depleted as well as standard blood termed QC base, low, medium, and high, as described previously.[15] The respective ASB activities measured in those sample by our assay were 0.20, 0.65, 3.4, and 8.8 μmol/(h L of blood). All values are blank subtracted using the measured activity of an assay cocktail incubated without a dried blood spot. Individual values for all samples are given in Table 2 and displayed in Figure S7 in the Supporting Information. Blank subtraction was performed as reported previously.[13]

Assay precision was calculated using triplicate analyses of DBS from a healthy control. The within-assay coefficient of variation (CV) was 1.1% (n = 3) while the interassay CV was 4.5% (n = 8 punches of the same DBS, avoiding the DBS perimeter). We also assayed 30 random newborn DBS by omitting ASB-S and found only background levels of ASB-P in all samples showing that DBS do not contain substances that interfere with the ASB assay.

Since cats have been used as an animal model in MPS-VI studies,[7,8] a comparison was made for animal samples by measuring the ASB activity with the solid-phase extraction method in feline DBS using 3 normal felines, 3 MPS VI carrier felines, and 3 MPS VI affected felines. The normal felines displayed an activity range of 160−360 μmol/(h L of blood). The carrier felines displayed values of 35−156 μmol/(h L of blood) and the MPS VI affected felines displayed values of 1−5 μmol/(h L of blood). Individual values for all samples are given in Table S1 and Figure S8 in the Supporting Information.

CONCLUSIONS

The new ESI-MS/MS assay of aryl sulfatase B activity in dried blood spots from humans and felines unambiguously distinguishes healthy individuals from affected ones. The assay uses a small amount of synthetic material (14.5 μg of substrate and 61 ng of internal standard per assay) and shows good linearity and interassay reproducibility. The workup procedure using solid-phase extraction of the product and internal standard generates virtually zero background but requires several liquid transfers, which is acceptable for a research laboratory. The workup procedure using liquid−liquid extraction of the product and internal standard provides very low background and, due to a minimum of liquid transfer steps, is suitable for large throughput analysis such as those performed in newborn screening laboratories.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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