Published in final edited form as:

Anal Chem. 2018 October 16; 90(20): 12168–12171. doi:10.1021/acs.analchem.8b03188.

Detection of Infantile Batten Disease by Tandem Mass Spectrometry Assay of PPT1 Enzyme Activity in Dried Blood Spots

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Abstract

A new tandem mass spectrometry (MS/MS)-based approach for measurement of the enzymatic activity of palmitoyl protein thioesterase I (PPT1) in dried blood spots (DBS) is presented. Deficiency in this enzyme leads to infantile neuronal ceroid lipofuscinosis (INCL, Infantile Batten disease, CLN1). The assay could distinguish between 80 healthy newborns and three previously diagnosed INCL patients. Unlike the fluorimetric PPT1 assay, the MS/MS assay does not require recombinant β -glucosidase. Furthermore, the assay could be easily combined with a TPP1 enzyme assay (for CLN2 disease) and can be potentially multiplexed with a large panel of additional lysosomal enzyme assays by MS/MS for newborn screening and post-screening analysis.

Introduction

Neuronal ceroid lipofuscinoses (NCLs) are a group of inborn errors of metabolism and constitute the most common type of hereditary neurodegenerative diseases of childhood with the highest incidence in Northern Europe and the U. S. (1:12,500).^{1–3} They are characterized by massive accumulation of auto-fluorescent storage material in various tissues, but especially in the brain, with clinical symptoms such as seizures, blindness, ataxia, epilepsy and progressive mental retardation. The most severe form of NCLs is the infantile type (INCL) with onset age of less than 2 years and a rapid progressive clinical course. INCL is a lysosomal storage disorder caused by mutations in the CLN1 gene on chromosome 1p32. The gene encodes palmitoyl protein thioesterase I (PPT1), a lysosomal enzyme that cleaves long-chain fatty acids (preferentially 14–18 carbon) from cysteine residues in certain proteins.⁴

Various therapies such as enzyme replacement therapy, gene therapy, neuronal stem cell therapy and small molecules therapy are being explored as potential NCLs treatments.^{5–8} Recently, FDA approved the first NCL therapy Brineura, an enzyme replacement therapy for CLN2.⁹ With the development of new therapies, and given the early onset and rapid progression of INCL, newborn screening for this disorder may someday be warranted.

The most common diagnosis of INCL is based on PPT1 enzyme assays in biological samples such as leukocyte, fibroblast and dried blood spot (DBS). Fluorimetric enzyme

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assay developed by van Diggelen et al.,¹⁰ uses the artificial substrate 4-methylumbelliferyl-6-thiopalmityl- β -D-glucoside (MUTPG), which upon the action of PPT1 followed by glycoside cleavage by β -glucosidase releases the fluorescent 4-methylumbelliferone for detection (Scheme 1). This assay has been used to measure PPT1 activities in newborn DBSs.¹¹

We have recently introduced a tandem mass spectrometry (MS/MS) assay of PPT1 activity in DBS using a synthetic palmitoylated oligopeptide substrate.¹² While the protocol showed robust performance, the assay conditions and the workup procedure did not allow for multiplexing with other lysosomal enzyme assays. Moreover, the PPT1 product undergoes partial proteolytic degradation. To address these issues, we developed and are presenting here a new method for the measurement of PPT1 activity in DBS by MS/MS analysis.

Materials and Methods

Materials.

Recombinant PPT1 was obtained as a gift from Mark Sands at Washington University, St. Louis. DBS from affected patients were obtained with IRB approval from previously diagnosed patients. Random newborn DBS were obtained as a generous gift from Prof. C. Auray-Blais (Unif. of Sherbrooke, Canada). All DBSs were stored at -20°C in sealed plastic bags stored in a close jar containing desiccant. MUTPG substrate was purchased from Cayman Chemical (Ann Arbor, MI, USA). TPP1 substrate and internal standard was prepared as described previously.¹⁷

Assay protocol.

A 3 mm DBS punch was eluted with 40 μL of phosphate-citrate buffer (0.4 M; pH = 4.5) in a 1.5 mL polypropylene microcentrifuge tube by agitation on an orbital platform shaker at 150 rpm for 45 min at room temperature. An aliquot of extract (30 μL) was transferred to a new 1.5 mL polypropylene microcentrifuge tube, and 15 μL of assay cocktail was added. Assay cocktail was octyl β -glucoside detergent (45 mM, Sigma-Aldrich-O8001), PPT1 substrate (1.2 mM) and internal standard (0.18 mM) in phosphate-citrate buffer (0.4 M; pH = 4.5). For the duplex assay the cocktail also contained TPP1 substrate (0.6 mM) and TPP1-IS (0.045 mM). The cocktails were prepared by speed-vac evaporation of a methanolic solution of the substrate(s), internal standard(s) and detergent and dissolving the residue in buffer. The assay mixture was incubated for 18 hr at 37°C in a thermostated air, orbital platform shaker at 250 rpm. The reaction was quenched by addition of 200 μL acetonitrile. The tube was vortexed and centrifuged, and 100 μL of supernatant was transferred to a well of a 96-well plate. Water (100 μL) containing formic acid (0.2%) and tris(2-carboxyethyl)phosphine hydrochloride, TCEP, (1.6 mM) was added, and the mixture incubated for 30 min at 37°C . The plate was covered with aluminum foil and placed in the autosampler chamber at 8°C in preparation for LC-MS/MS analysis. It is also possible to do all steps of the assay in 96-well, microtiter plates.

LC was carried out using a Waters Acquity binary solvent pump system with a CSH, C18, 1.7 μm , $2.1 \times 50\text{mm}$ column (Waters, 186005296) and a CSH, C18, 1.7 μm guard column

(Vanguard, 186005303). The solvent program was 80% A (water, 0.1% formic acid)/20% B (acetonitrile, 0.1% formic acid) to 34% A/66% B over 1.2 min, then jumped to 99% B and held for 1.30 min, then to 80% A (flow rate 0.4 mL/min). The total run time was 2.5 min. All LC solvents were Optima grade from Fisher Scientific. The LC eluent was diverted to waste in the time region 0–0.3 min (during void volume elution) and 1.1–1.4 min (when the detergent eluted) to minimize contamination of the MS/MS electrospray source. MS/MS was carried out with a Waters Xevo TQ tandem-quadrupole instrument. Acquisition parameters are provided in Table S3 of the supporting information.

To obtain the enzymatic activity, the product-to-internal standard ion count area ratio was multiplied by the micromoles of internal standard in the assay, and this number was divided by the incubation time (18 hr) and the volume of blood in a 3 mm DBS punch (3.2 μ L).

Results

The published fluorimetric assay for DBS makes use of the artificial substrate MUTPG.¹¹ PPT1 hydrolyzes the thioester to form the free fatty acid and the 4-methylumbelliferyl-glycoside of 6-thioglucoase. Endogenous β -glucosidase in the DBS releases free 4-methylumbelliferone from the PPT1 product; however, exogenous β -glucosidase is typically added to the assay to ensure that this conversion is complete.^{10,11}

Initially we explored a set of potential novel substrates for PPT1, and these are shown in Table 1. Compounds **1** and **2** bear a bis-amide unit that is found in many of our previously developed substrates for lysosomal enzymes; this unit is well protonated in the gas phase during the MS/MS assay. Compound **3** is an analog of our previously reported PPT1 substrate and contains an acetamido group at the end of the fatty acyl chain. This would allow the released fatty acid product to be detected by MS/MS. Detection of the peptide product is possible, but this does not allow multiplexing with other lysosomal enzymes. Compounds **5–7** are analogs of MUTMG that contain the bis-amide unit found in many of our other lysosomal enzyme substrates. Compounds **1–4** did not show specificity towards PPT1 enzyme and were cleaved by other thioesterase in the blood. Thioesters **5–7** displayed lower PPT1 activity compared to MUTMG and did not offer any other advantages over MUTMG. Thus, we focused our assay development on the previously reported PPT1 substrate MUTMG.

In the MS/MS assay, the preferred PPT1 product is the 4-methylumbelliferyl-glycoside of 6-thioglucoase since 4-methylumbelliferone does not fragment well in the mass spectrometer. The MS/MS assay uses octyl-glucoside as the detergent instead of Triton X-100 used in the fluorimetric assay. The former is a competitive substrate for endogenous β -glucosidase such that the PPT1 product does not undergo further conversion in the assay with DBS.

We found out that use of an aqueous extract of DBS rather than addition of a DBS punch directly to the assay cocktail leads to significantly higher PPT1 activity. Presumably, miscellaneous lipids in DBS that extract into the detergent-containing buffer act as competitive inhibitors and/or substrates for PPT1. Such lipids should not be extracted from the DBS into an aqueous buffer lacking detergent.

The MUTPG substrate has a 16-carbon fatty acyl chain. Previous studies, however, suggested that the optimum PPT1 activity is obtained with a fatty acyl chain length of 14 carbons.^{13,14} We synthesized the 14-carbon analogue of MUTPG, 4-methylumbelliferyl-6-thiomyristoyl- β -D-glucoside (MUTMG, Figure 1) and compared its enzymatic activity with that of MUTPG. In our MS/MS assay at pH 4, 4.5 and 5.0, higher PPT1 activity by 1.3-, 1.6- and 3.9-fold, respectively, was seen with the 14-carbon substrate versus the 16-carbon substrate (Figure S1, Supporting Information).

The optimum pH for PPT1 activity is known to vary with the substrate and the other assay conditions.^{4,15} For the MUTPG substrate the pH optima was reported to be in the range of 4 to 5.^{4,16} Under our assay conditions, enzymatic activity was higher at pH 4.5 than at pH 4 or 5 with both MUTPG and MUTMG substrates (Figure S1, Supporting Information). Thus, we settled on the 14-carbon substrate, MUTMG, at pH 4.5 for all subsequent studies. The MS/MS assay was also carried out in the presence of an internal standard, which is chemically identical to the PPT1 product but isotopically different (Figure 1). The use of this internal standard accounts for all losses of PPT1 product due to downstream enzymes and/or absorption to the walls of containers. It also accounts for suppression of ionization in the electrospray source of the mass spectrometer.

The incubation time for the assay was set to 18 h; PPT1 enzymatic product increases linearly with the incubation time up to 18 h (Figure S2, Supporting Information). Shorter incubation times are also possible if more convenient.

PPT1 activity was measured using a healthy adult DBS over the MUTMG substrate concentration range of 0–400 μ M. The data fit well to the standard Michaelis-Menten equation yielding $K_M = 105$ μ M and $V_{max} = 194$ μ mol/hr/L (Figure S3, Supporting Information). All subsequent studies were carried out with 400 μ M MUTMG substrate.

PPT1 product formation as a function of the amount of the enzyme showed an approximate linear correlation (Figure S4, Supporting Information). These studies were carried out by adding increasing amounts of recombinant PPT1 to DBS extract that had been heat treated at 90 °C for 3 min to destroy endogenous PPT1.

Clinical Sample Analysis

We measured the PPT1 activity in DBS from 80 random newborns and 3 previously diagnosed INCL patients with the optimized assay conditions. The results are compiled in Table S1 (Supporting Information) and are depicted in Figure 2. The blank in which DBS was replaced with a filter paper gave an activity of 2.5 μ mol/hr/L. After blank correction, unaffected newborns showed activities of 13–314 μ mol/hr/L with an average of 64 μ mol/hr/L. The PPT1 activities of INCL patients, after blank correction, were measured to be 1.1–1.8 μ mol/hr/L. It should be noted that whereas the patients DBSs were less than 20 months old, the random newborn DBSs were more than six years old and are thus likely to give a lower limit of the PPT1 activity measured in fresh DBS from healthy newborns.

We also developed a duplex assay to measure PPT1 activity together with the lysosomal enzyme tripeptidyl peptidase I (TPP1). Deficiency in TPP1 causes the late infantile form of

NCL (LINCL, CLN2). The previously reported TPP1 substrate and internal standard¹⁷ were added to the PPT1 assay cocktail. The cocktail was incubated with DBS extracts from healthy adults or INCL patients. The PPT1 and TPP1 activities were measured simultaneously using the appropriate single reaction monitoring MS/MS channels for each product and internal standard. Table S2 (Supporting Information) provides the duplex assay results. The blank in which DBS was replaced with filter paper gave a PPT1 activity of 3.1 $\mu\text{mol/hr/L}$ and a TPP1 activity of 0.6 $\mu\text{mol/hr/L}$. After blank correction, the four healthy adults showed PPT1 activities of 16–67 $\mu\text{mol/hr/L}$ and TPP1 activities of 109–212 $\mu\text{mol/hr/L}$. The three INCL patients showed PPT1 activities of 0.8–0.9 $\mu\text{mol/hr/L}$ and TPP1 activities of 128–220 $\mu\text{mol/hr/L}$.

Discussion

The new PPT1 assay utilizes a substrate that can be used for both fluorimetric and MS/MS assays. The fluorimetric assay would require that the octyl β -glucoside detergent be replaced with Triton X-100 and that exogenous β -glucosidase be added to ensure complete conversion of the immediate PPT1 product to free 4-methylumbelliferone. In this study we combined the PPT1 assay with our previously reported assay for TPP1. It should also be possible to multiplex both of these assays with our full panel of MS/MS assays of several lysosomal enzymes. The development of the full multiplex is in progress and will be reported elsewhere. These assays use gradient elution liquid chromatography in combination with MS/MS. This adds very little additional complexity and cost compared to flow injection-MS/MS assays since a solvent delivery pump is needed for the latter and an additional pump is needed for gradient elution liquid chromatography.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements.

This work was supported by a grant from the National Institutes of Health ((R01 DK067859).

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Figure 1.
Structures of the PPT1 substrates and the internal standard.

Figure 2.
PPT1 activity in DBS measured by MS/MS with MUTMG substrate.

Scheme 1.

Table 1.

Structures of the tested potential PPT1 substrates.