Fluorimetric assay with a novel substrate for quantification of galactocerebrosidase activity in dried blood spot specimens

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**ABSTRACT**

**Background:** Decreased galactocerebrosidase (GALC) enzyme activity is causative for Krabbe disease, a lysosomal storage disorder with devastating neurodegenerative consequences. Quantitative fluorimetric assays for GALC activity in isolated blood and skin cells have been described; however, no such assay has been described using dried blood spot (DBS) specimens.

**Methods:** GALC enzyme activity was measured quantitatively using fluorescence from a novel glycosidic substrate: carboxy derived from 6-hexadecanoylamino-4-methylumbelliferone. GALC activity was demonstrated on newborn DBS specimens, known Krabbe disease patient specimens, proficiency testing and quality control samples.

**Results:** We present data on characterization of the novel substrate and assay, including pH optimization and enzyme kinetics using a fluorimetric profile. Single and multi-day precision analyses revealed tight analytical measurements with %CV ranging from 5.2% to 14.1%. GALC enzyme activity was linear over the range of 0.31 - 12.04 μmol/l/h with a limit of detection of 0.066 μmol/l/h. Our results with this assay show a clear discrimination between GALC activities in samples from Krabbe disease patients versus presumed normal newborn samples.

**Conclusions:** A fluorimetric assay for GALC enzyme activity measurement on dried blood spot specimens is feasible. Improvements to the assay including novel substrate design, increased substrate concentration and removal of sodium chloride maximize the specificity of the assay and minimize interference from β-galactosidase.

**Abbreviations:** GALC, galactocerebrosidase; NBS, Newborn screening; DBS, Dried blood spot; QCBP, Quality control base pool; QCL, Quality control low sample; QCM, Quality control medium sample; QCH, Quality control high sample; PT, Proficiency testing; MUG, 4-methylumbelliferyl-β-D-galactopyranoside substrate; HMU, 6-hexadecanoylamino-4-methylumbelliferone; HMUG, 6-hexadecanoylamino-4-methylumbelliferone β-D-galactopyranoside substrate; CHMUG, 3-Carboxy 6-hexadecanoylamino-4-methylumbelliferone β-D-galactopyranoside substrate; CHMU, 3-Carboxy 6-hexadecanoylamino-4-methylumbelliferone; RFU, Relative fluorescence units; rhGALC, Recombinant human galactocerebrosidase; rhβ-gal, Recombinant human β-galactosidase; NEH, Non-enzymatic hydrolysis; CLSI, Clinical and Laboratory Standards Institute; LoD, Limit of detection; LoB, Limit of the blank; LoQ, Limit of quantitation.

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1. Introduction

The lysosomal enzyme galactocerebrosidase (GALC; EC 3.2.1.46) hydrolyzes glycosidic bonds of several glycosphingolipids, including galactose from galactosylsphingosine (psychosine), and is essential to prevent the toxic accumulation of psychosine in the body. Relative to other lysosomal enzymes, GALC protein is expressed at very low levels in blood and tissues [1]. It is also highly hydrophobic and consequently, readily aggregates with itself and other hydrophobic proteins [2]. Extensive characterization of the enzyme structure and kinetics for synthetic GALC substrates revealed relatively slow $V_{\text{Max}}$ (46.6 ± 1.6 nmol min$^{-1}$ μg$^{-1}$) and $k_{\text{cat}}$ (57.1 s$^{-1}$) for GALC under optimal conditions [3]. Specificity of GALC measurement is also complicated by the high degree of structural similarity between its synthetic substrate and synthetic substrates for other galactosidase enzymes such as β-galactosidase (β-gal; EC 3.2.1.23). Both substrates are composed of a galactose moiety attached to an aglycone dye via a glycosidic linkage; meticulous optimization of assay conditions is, therefore, necessary to maximize specificity of the respective enzymes [4,5]. Together, these features have complicated efforts to develop sensitive and specific assays to quantify GALC activity in human biological specimens.

Mutations in the GALC gene, and resultant deficiency in GALC enzyme activity, lead to Krabbe disease – a rare but devastating neurodegenerative disease that typically presents in infancy and progresses rapidly [6–8]. Krabbe disease is treated with hematopoietic stem cell transplantation when diagnosed before the onset of symptoms. Several public health laboratories in the U.S. are already performing newborn screening (NBS) for this condition [9] While fluorimetric assays for the measurement of GALC activity have been reported using fibroblasts [10] and leukocytes [11,12], these methods have not been demonstrated on dried blood spots (DBS), the standard specimen for NBS in public health laboratories. Tandem mass spectrometry methods for GALC activity measurement using DBS are also available but require protracted sample processing steps plus significant investment for equipment capital and maintenance, which may limit widespread adoption of this method by NBS programs [13–15].

In this report, we demonstrate a rapid, microtiter plate fluorimetric assay for measuring GALC enzyme activity in DBS specimens using a novel substrate. The substrate for this assay has β-galactose conjugated with a fluorogenic derivative of 6-hexadecanoyl-4-methylumbelliferone with a hydrophobic group. We present data including fluorescence characterization of this novel substrate, pH optimization, enzyme kinetics, and extensive analytical characterization data including single and multi-day precision, accuracy, linearity, and specificity of the assay for GALC over β-galactosidase. The resulting assay can be performed in microtiter plates with standard lab equipment to determine GALC enzyme activity in dried blood spots or with whole blood. Obtaining fresh whole blood samples for a direct comparison can be challenging, especially from untreated individuals. Therefore, we proceeded with the DBS assay as run in public health laboratories. This assay would determine GALC activity in DBS to screen for enzyme deficiency and all results should be confirmed with secondary testing to make a diagnosis or assess the efficacy of treatment.

2. Materials and methods

2.1. Dried blood spot samples

Archived, de-identified newborn DBS samples were obtained from the Missouri State Public Health Laboratory; these specimens were collected prior to March 2015 and therefore do not require informed consent for use [16]. Samples were obtained as individual punches (3.2 mm diameter) from DBS cards of presumed normal newborns. Archived, de-identified DBS from 10 affected Krabbe disease patients were obtained from the Legacy of Angels Foundation. Quality control (QC) DBS were prepared in-house by the method previously described by De Jesus and colleagues [17]. Briefly, packaged red blood cells obtained from Tennessee Blood Service (Memphis, TN) were washed with saline, and then mixed with heat-inactivated and charcoal-stripped serum (SeraCare Life Sciences, Gaithersburg, MD) to make a base pool (QCBP) with 50% hematocrit. The QCBP has low to no enzyme activity as assessed previously [17]. QC high (QCH) DBS samples, with high enzyme activity, were prepared from cord blood units (Carolinas Cord Blood Bank, Durham, NC) and adjusted to 50% hematocrit. QC DBS with low (QCL) and medium (QCM) enzyme activities were generated by combining the QCBP and QCH pools. GALC deficient proficiency testing (PT) DBS, used as control samples with known enzyme deficiency, were obtained from the Centers for Disease Control and Prevention (CDC, Atlanta, GA) and stored with a desiccant, sealed in a Ziploc bag at -80°C. All clinical samples were stored at -80°C upon receipt and run within three months of sample receipt. Under these timelines and storage conditions, we did not expect any significant changes in GALC activity [17].

2.2. Reagents

Sodium acetate (anhydrous), sodium chloride, sodium citrate trihydrate, citric acid monohydrate, 4-methylumbelliferone sodium salt, 4-methylumbelliferyl-β-D-galactopyranoside (MUG), sodium taurocholate hydrate, sodium bicarbonate, glacial acetic acid, dimethyl sulfoxide (DMSO), ethylene glycol tetraacetic acid tetrasodium salt (EGTA), Tween 20, Triton X-100, 96-well flat-bottom black polystyrene half-area microtiter plates, and clear adhesive plate sealers were obtained from Millipore Sigma Corp. (St. Louis, MO). Molecular biology grade water, 96-well clear round-bottom microtiter plates, and adhesive aluminum plate sealers were obtained from Fisher Scientific (Pittsburgh, PA). 6-hexadecanoylamino-4-methylumbelliferone (HMU) was purchased from Glycosynth, Ltd. (Warlington, Cheshire, England) and used as a fluorescence standard. A modified carboxy-derivative of 6-hexadecanoylamino-4-methylumbelliferyl-β-D-galactopyranoside (HMG) was synthesized at CiVentiChem, Inc. (Cary, NC); this substrate was the 3-carboxy 6-hexadecanoylamino-4-methylumbelliferone β-D-galactopyranoside substrate (CHMUG). A carboxy-derivative of HMU (CHMU) was also synthesized for spectroscopic comparison (CiVentiChem, Inc.) with the unmodified 6-HMU fluorophore. Recombinant human galactocerebrosidase (rhGALC) and recombinant human β-galactosidase (rhβ-gal) were obtained from R&D Systems, Inc. (Minneapolis,
Sample extraction solution was prepared by diluting Tween 20 in molecular biology grade water to 0.1% w/v. The stop solution consisted of 500 mmol/l sodium bicarbonate (pH 10.7) with 0.15% (w/v) Triton X-100 and 0.01% (w/v) Tween 20. The GALC substrate solution was prepared by adding sodium taurocholate (1.5% w/v), Tween 20 (0.01% w/v), EGTA (2.5 mmol/l) and GALC substrate (0.5 mmol/l) to 100 mmol/l sodium acetate buffer (pH 4.3). The GALC substrate was obtained as a yellowish powder and was dissolved in DMSO to a stock concentration of 100 mmol/l.

2.3. Spectroscopic analysis

The absorbance spectra of the HMU standard and the modified carboxy-HMU (CHMU) were compared to determine similarities between the two fluorophores. The HMU standard and the CHMU were both dissolved in stop solution to a final concentration of 0.5 μmol/l (E = 13,300 M–1 cm–1 at 390 nm) and measured using an Agilent 8453 UV–visible spectrophotometer (Agilent Technologies, Santa Clara, CA). Seven hundred microliters of each solution were added to quartz cuvettes (Thorlabs Inc., Newton, NJ) and the absorbance characteristics were analyzed at wavelengths ranging from 300 – 600 nm that included the expected absorbance maxima at 390 nm, in increments of 1 nm. The experiment was repeated three times with measurements taken in triplicate for each solution. The fluorescence emission spectra for 0.5 μmol/l of HMU standard and CHMU were also analyzed using the BioTek Synergy H1 monochromator-based multi-mode plate reader (Biotek, Winooski, VT). Seventy microliters of each solution were added in triplicate to individual wells of a black half-area 96-well microtiter plate. Fluorescence was measured using an excitation wavelength set to 390 (±9) nm and emission spectra obtained at wavelengths ranging from 420 – 600 nm.

2.4. GALC enzyme assay

2.4.1. DBS sample extraction

To extract galactocerebrosidase enzyme from the DBS samples, one punch (3.2 mm) from each DBS was placed in individual wells of a clear, round-bottom, 96-well microtiter plate. Sample extraction solution (100 μl) was added to each sample well, the plate was covered with a clear adhesive sealer to prevent evaporation and then incubated on a plate-shaker (600 rpm) at room temperature (RT) for 30 min.

2.4.2. Enzyme activity measurement

Enzyme activity was determined by adding 10 μl of DBS extract to 10 μl of the GALC substrate solution, which was varied according to the values in Fig. 2. We analyzed several substrate concentrations in order to identify the activity, Vmax and Km of the GALC enzyme. The optimal pH was determined to be pH 4.3. Experiments were performed in individual wells of black, half-area 96-well microtiter plates. Ten microliters of sample extraction solution (without DBS) were added to four replicate control wells to measure any back-fluorescence due to non-enzymatic hydrolysis (NEH) of the substrate. Extracts from quality control DBS with low (QCL) and high (QCH) enzyme activity were loaded in duplicate wells to serve as positive and/or negative controls for enzyme activity. The first column (8 wells) of the microwell plate was left empty for use with the HMU standards to generate a calibration curve. The plates were covered with adhesive aluminum plate-sealers and briefly centrifuged at 400 × g for 30 s. A moistened Kimwipe (thin paper towel; Kimberly-Clark Corp., Irving, TX) was placed flat on top of the sealed plate and the plate was firmly wrapped in a piece of aluminum foil to create a humidified enclosure to prevent excess evaporation. The wrapped plate was incubated for 17 h (overnight) in an incubator at 37 °C. The low catalytic turnover (kcat) of the GALC enzyme [3] necessitated an overnight incubation. The reaction was terminated at the end of the overnight incubation by adding 50 μl of stop solution to each sample well and each of the four replicate control wells (non-enzymatic hydrolysis control; NEH). Seventy microliters of each concentration of the 6-HMU standards (0.0156, 0.0313, 0.125 and 0.5 μmol/l) were added to wells in the first column, in duplicate. The fluorescence of the plate, measured as relative fluorescence units (RFU), was read in a BioTek Synergy HTX microtiter plate reader with 400 (±15) nm excitation and 485 (±20) nm emission filters.

Enzyme activity, expressed as μmol of 6-HMU generated per liter of blood per hour of incubation (μmol/l/h), was calculated using the following formula:

\[
\frac{RFU_{\text{Sample}} - RFU_{\text{NEH}}}{\text{slope} \times RFU_{\text{μmol/l}}} \times \frac{\text{vol}_{\text{total extract}} \times \mu l}{\text{vol}_{\text{DBS Punch}} \times \mu l} \times \frac{\text{vol}_{\text{final reaction}} \times \mu l}{\text{time} \times \text{hr}}
\]

The average fluorescence in the NEH wells was subtracted from the fluorescence measured in the individual sample wells to obtain the background subtracted fluorescence value representative of enzymatic hydrolysis of the substrate. This background subtracted fluorescence value was compared to the fluorescence calibration curve obtained from the 6-HMU dilution set. The μmoles of HMU released by enzymatic hydrolysis of the substrate were obtained by dividing the background subtracted fluorescence value with the slope of the calibration curve to obtain the relative μmoles of HMU. The resulting value was adjusted for the volume of blood tested in the individual well (assume 3.1 μl for a 3.2 mm punch of DBS extracted into 100 μl, and further diluted 1:7 in the final reaction volume [18]) and the number of hours incubated (17 h) to obtain enzyme activity (expressed as μmol/l/h).
2.5. GALT assay optimization and analytical characterization

2.5.1. pH optimization

The GALT substrate solution was prepared at pH 3.4, 3.7, 4.0, 4.3, 4.6, 4.9, 5.2 and 5.5 in 100 mmol/l acetate buffer to determine the optimal pH conditions for galactocerebrosidase activity measurement using a substrate concentration of 0.5 mM.

2.5.2. Enzyme kinetics

The GALT substrate was tested at a range of concentrations (0.016, 0.031, 0.063, 0.125, 0.250, and 0.5 mmol/l), diluted 1:1 in the substrate buffer, to study the kinetics of the enzyme at a pH of 4.3 for optimal conditions.

2.5.3. Sodium chloride optimization

To determine the optimal chloride concentration for GALT enzyme activity measurement, the GALT assay was performed both with and without sodium chloride (150 mmol/l or 0 mmol/l sodium chloride in the assay buffer). 150 mmol/l sodium chloride was chosen as this is the optimal salt concentration for the β-galactosidase (MUG) assay, described in the following section. Both salt conditions were tested on QCH and PT DBS samples using previously described methods or on recombinant enzyme samples as follows. Pooled extract from 15 punches of QCBP was used as a biological matrix to dilute rhGALT and rhβ-gal enzymes to concentrations of 0.2 μg/ml and 0.68 μg/ml, respectively. Each enzyme concentration was tested in duplicate in each assay setup (±NaCl). A calibration curve was prepared and the enzyme activities calculated as described above for the GALT assay.

2.5.4. GALT assay specificity

To characterize the specificity of our GALT assay conditions for GALT relative to β-galactosidase, we compared the activity of QC and PT DBS using both the GALT assay and a specific β-galactosidase (MUG) assay. The MUG assay was based on the method of Civallero et al. [19]. Briefly, a substrate solution with 1.5 mmol/l 4-methylumbelliferyl-β-D-galactopyranoside (MUG) was prepared in 100 mmol/l sodium citrate/200 mmol/l sodium phosphate (Na2HPO4) buffer (pH 4.0) and 150 mmol/l sodium chloride. A stop solution was prepared as described earlier for the GALT assay. QCH and PT DBS were extracted in sample extraction solution as described above. These extracts were tested in duplicate using both the β-galactosidase assay [19] and the GALT assay, described above, by adding 10 μl of each substrate solution (MUG or GALT substrate). Four replicate wells of the NEH (negative) control were also set up for each assay. The plates were sealed as described above and incubated at 37 °C. The enzyme reaction was terminated after 17 h for both the β-galactosidase assay and the GALT assay using 50 μl of stop solution.

2.5.5. Linearity determination

The linear range for the GALT assay was determined using rhGALT enzyme diluted in QCBP at concentrations ranging from 0 to 40 pg/μl and tested according to the Clinical and Laboratory Standards Institute (CLSI) guidelines EP6-A [20]. Recombinant enzyme was utilized in order to ensure we examined and characterized the entire clinically relevant range. Six replicates of each dilution were tested. Enzyme activities were calculated and expressed in μmol/l/h and analyzed statistically to determine the best regression fit (linear or non-linear) for the data.

2.5.6. Analytical sensitivity determination

The lower limit of detection (LOD) and the limit of quantitation (LOQ) were determined based on CLSI guidelines EP17-A [21]. The limit of the blank (LOB) was determined by testing QCBP extract as the blank sample with no analyte (no enzyme activity), and the LOD was determined by testing QCL extract as the sample with low levels of analyte (low enzyme activity). Two different operators each performed an independent run with 40 replicates of each QCBP and QCL for a total of 80 data points representing no and low levels of enzyme activity. The LOB and LOD were calculated using α and β = 5%, where α and β represent the Type I (false positive measure of analyte) and Type II (false absence of analyte) errors, respectively. LOQ was determined by testing a pool of extracts from presumed normal DBS samples (with presumed normal levels of enzyme activity) diluted with a pool of QCBP extracts. Eight dilutions were prepared ranging from 0 (undiluted QCBP) to 1:10 (QCBP in DBS). The complete dilution set was distributed evenly between these QC levels. The mean activity and precision were calculated for each dilution.

2.5.7. Precision determination

Precision was measured based on CLSI guidelines EP5-A2 [22]. Intra-assay and inter-assay variability were determined by measuring enzyme activity using the GALT enzyme assay described above over 5 non-consecutive days by two different operators. Each day, the operator performed an independent run with 8 replicates of each of the quality control samples (QCL, QCJM, and QCH), no enzyme, and low enzyme activity samples, for a total of 80 data points each day. The intra-assay and inter-assay variability were calculated to determine inherent imprecision in the assay.

2.6. Statistical analysis

All statistical analyses and comparisons were carried out using GraphPad Prism software (ver. 6.07).
Fig. 1. Absorbance and fluorescence profiles of HMU and CHMU. (A) Chemical structures of the non-fluorescent substrates (top), fluorescent products (middle), and final phenolate products (bottom) for HMU (left) and CHMU (right). (B) Absorbance of HMU (solid green line) and CHMU (broken red line) were measured at a range of wavelengths (300 - 600 nm) in 1 nm increments. The broken, vertical black line indicates the excitation wavelength (400 nm) used for fluorescence measurements in the GALC assay. The shaded area represents the 30 nm bandwidth of the excitation filter. (C) The fluorescence of HMU (solid green line), CHMU (broken red line), and the assay blank (stop solution; solid black line) were measured at a range of emission wavelengths (420–600 nm) in increments of 5 nm at a fixed excitation wavelength of 390 nm. The vertical, broken black line indicates the emission wavelength (485 nm) used to measure the fluorescence in the GALC assay. The shaded area represents the 40 nm bandwidth of the emission filter used.
3. Results

3.1. Absorbance and fluorescence profiles of HMU and CHMU

The substrates previously used for GALC activity measurement are hydrophobic [10] and are not compatible with high throughput testing due to the complex preparation requirements [10,23]. We, therefore, developed a novel, water-soluble substrate using a modified carboxy-derivative of a 6-hexadecanoylamino-4-methylumbelliferyl galactopyranoside (CHMUG). Fig. 1A illustrates the chemical structures of HMUG (left) and its modified form, CHMUG (right), as well as the modifications that each substrate undergoes following exposure to enzyme or stop solution. Briefly, the aglycone dye 7-hydroxy 6-hexadecanoyl-4-methylumbelliferone is non-fluorescent while the phenol group remains protected either as a glycoside or through protonation. Hydrolysis of the glycosidic linkage between the sugar and the aglycone under low pH conditions generates the fluorophore, which becomes fluorescent following termination of the enzymatic reaction by addition of a high pH stop solution. Assay conditions and substrates were designed to provide maximum assay sensitivity to separate the activity values between no enzyme samples and normal enzyme samples.

Although the substrate was designed as a carboxy-HMUG, the calibrants for the standard curve used to calculate enzyme activity were prepared using the more readily available HMU salt. Therefore, we analyzed CHMU, the product of the GALC reaction, to compare its absorbance and fluorescence properties to that of HMU at equal concentrations (0.5 μmol/l) and confirmed that the two compounds exhibit similar fluorescence characteristics under the described assay conditions. The absorbance (Fig. 1B) and fluorescence (Fig. 1C) profiles of HMU and CHMU are highly similar. HMU and CHMU have absorbance maxima at 393 nm and 391 nm, respectively, with their absorbance profiles showing a significant overlap over a range of wavelengths (Fig. 1B). In particular, the absorbance is identical at 400 nm (±15 nm) (grey column, Fig. 1B), which is the excitation wavelength that is used to measure fluorescence from the release of CHMU during the enzymatic hydrolysis of the GALC substrate. The fluorescence profiles of the two compounds show some variation when excited at a fixed wavelength of 390 nm, but exhibit identical fluorescence at 485 nm (grey column, Fig. 1C), which is the emission wavelength utilized for all subsequent experiments. Although maximal absorbance for CHMU occurs at 390 nm (Figs. 1B), 400 nm excitation is used in all subsequent experiments due to filter availability on the BioTek Synergy HTX plate reader. No measurable difference in fluorescence emission (at 485 nm) was found for excitation at 400 nm (BioTek Synergy HTX) versus 390 nm (BioTek Synergy H1) (Fig. 1C).

3.2. Optimization of assay conditions

The GALC assay was carefully optimized to ensure robust performance from the small amount of enzyme present in DBS and to minimize interference from β-galactosidase. We also selected an optimal substrate concentration (based on the GALC enzyme Km) and performed a careful evaluation of β-galactosidase activity to ensure maximum specificity of the optimized GALC assay.

3.2.1. pH optimization

Lysozomal enzymes operate at an acidic pH in their native environment and each enzyme requires an optimal pH for efficient substrate cleavage [24]. To find the pH with highest activity, we performed the GALC assay under a wide range of pH conditions. A pooled extract from presumed normal DBS punches was used as the enzyme source and the sample was tested using GALC substrate solutions adjusted to pH 3.4, 3.7, 4.0, 4.3, 4.6, 4.9, 5.2, and 5.5. GALC enzyme showed highest activity at pH 4.3 (Fig. 2A), which was used for all subsequent testing.

![Fig. 2. pH optimization and Km/Vmax determination.](image-url)
3.2.2. \( K_m \) and \( V_{max} \) determination

The Michaelis-Menten kinetics of the GALC enzyme assay was determined using pooled extract from 47 normal DBS punches as the GALC enzyme source. Enzyme activity was assessed using substrate concentrations of 0.02, 0.03, 0.06, 0.13, 0.25 and 0.5 mmol/l and a 17 h incubation of the reaction mixture. The \( K_m \) for the reaction was determined to be 0.229 mmol/l based on the non-linear regression analysis of the enzyme kinetics (Fig. 2B). Based on these findings, a substrate concentration of 0.5 mmol/l (slightly more than twice the \( K_m \)) was used for all subsequent analyses to ensure that substrate concentration would not be limiting during the incubation period. The 17 h incubation time for the assay was chosen based both on theoretical calculations using \( V_{max} \) and \( K_m \) and from comparisons of measured activity following incubation times ranging from 6 to 24 h (data not shown). Using the final assay conditions outlined in this report, the 17 h incubation time maximizes the fluorescence output of the assay without introducing bias associated with excess incubation times [25].

3.2.3. Buffer optimization

Chlorides, including sodium chloride, that are present in the assay buffer can increase nonspecific signal from \( \beta- \)galactosidase [26, 27]. To determine the effect of sodium chloride on our GALC enzyme assay, the substrate cocktail was prepared with and without sodium chloride. As shown in Fig. 3A, addition of 150 mmol/l sodium chloride in the substrate solution caused a significant increase in enzyme activity measured in both the QCH and deficient PT samples. Furthermore, when recombinant GALC and \( \beta- \)gal enzyme samples were tested (Fig. 3B), sodium chloride (150 mmol/l) caused significant increases in both samples compared to 0 mmol/l sodium chloride. The greatest increase in activity was seen with the rh\( \beta \)-gal enzyme, which showed 55 times greater activity in the presence of sodium chloride. Enzyme activity measured from rhGALC, by contrast, increased only 2.4 fold in the presence of sodium chloride. These findings show that the presence of sodium chloride in the reaction mixture leads to an increase in non-specific \( \beta- \)galactosidase activity measurement. Sodium chloride was, therefore, omitted in the final GALC assay protocol. All data presented here, with the exception of Figs. 3 and 4A, were generated in the absence of sodium chloride.

3.3. Analytical performance

3.3.1. Analytical specificity

GALC and \( \beta- \)galactosidase are both present in the same lysosomal location, have optimal activity around pH 4.0, and have similar substrate specificities [4]. To determine the specificity of our CHMUG substrate, we analyzed quality control high (QCH) DBS samples and a proficiency testing (PT) DBS sample using both our fluorimetric GALC assay and the standard \( \beta- \)galactosidase MUG assay. QCH samples are expected to have normal enzyme activity, while PT deficient samples should have reduced GALC activity. In light of our previous findings, which showed that sodium chloride has a much stronger activating effect on \( \beta- \)galactosidase compared to GALC (Fig. 3), we included physiological concentrations of sodium chloride in the \( \beta- \)galactosidase assay (Fig. 4A) and did not use sodium chloride in the CHMUG GALC assay (Fig. 4B). As shown in Fig. 4A, the \( \beta- \)galactosidase MUG assay measured similar enzyme activity in the QCH and GALC PT samples; this result was expected and indicates normal \( \beta- \)galactosidase activity in these two samples. By contrast, the same samples tested with the fluorimetric GALC assay exhibited very different activity levels: high activity in the QCH sample and very low activity in the PT deficient sample (Fig. 4B). The latter finding is consistent with expectations for a sample that is deficient in the GALC enzyme. Together, these results demonstrate that endogenous \( \beta- \)galactosidase does not cleave the CHMUG substrate in our fluorimetric GALC assay without sodium chloride and illustrate the high degree of specificity of our assay conditions and the CHMUG assay.

Fig. 3. Buffer optimization. Pooled extracts from QCH and PT dried blood spots (A) or recombinant human GALC and \( \beta- \)galactosidase (B) were tested for GALC activity in the presence (grey) or absence (black) of 150 mmol/l NaCl to assess the effect of salt on measured enzyme activity. The graphs show mean enzyme activities for each sample tested in duplicate, with error bars representing the standard deviations from the mean.
substrate for the GALC enzyme.

### 3.3.2. Analytical sensitivity

Analytical sensitivity of the GALC assay was established by measuring the limit of the blank (LoB) of QCBP sample extracts, the limit of detection (LoD) of QCL sample extracts, and the LoQ of presumed normal patient DBS extracts. The mean of the blank sample was $4.0 \times 10^{-5}$ μmol/l/h with a standard deviation (SD) of 0.016 μmol/l/h (n=96). For $\alpha = 5\%$, the LoB was calculated to be 0.027 μmol/l/h. The mean of the low sample (QCL) was 0.2 μmol/l/h with a SD of 0.021 μmol/l/h (n=96). For $\beta = 5\%$, the LoD was calculated to be 0.066 μmol/l/h. The assay was sensitive enough to detect enzymatic activity for all dilutions of the presumed normal DBS extracts. The LoQ was determined to be the lowest concentration that was tested, that is above the LoD, 0.31 μmol/l/h and 10.8% CV (n=14). Further testing could show it to be lower but was not performed.

### 3.3.3. Linearity

The linear range of the fluorimetric GALC assay was determined by testing a range of dilutions of the GALC enzyme (0–40 pg/μl) prepared using recombinant human GALC (rhGALC) enzyme diluted in a QCBP matrix solution. The plot of enzyme activity as a function of enzyme concentration (Fig. 5) showed a linear correlation ($R^2=0.936$). The linear regression model was a significantly better fit than the nonlinear higher order regression models (second-order coefficient, $p=0.613$ and third-order coefficient, $p=0.13$) demonstrating that the assay was linear along the entire activity range tested (0.31–12.04 μmol/l/h). Similar results were found in a range of GALC enzyme prepared by serial dilution of QCH DBS extract (data not shown). With a substrate concentration of 0.5 mM, the assay has a linear correlation with $R^2$ of 0.98 over a 20-h time course (Fig. 5B).

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**Fig. 4. Specificity of GALC assay conditions.** (A–B) Pooled extracts from quality control high (QCH; grey) and proficiency testing deficient (PT; black) samples were tested using two different assay conditions optimized to measure activity of β-galactosidase (A) or GALC (B). The graphs show mean enzyme activities for each sample tested in duplicate, with error bars representing the standard deviations from the mean.

**Fig. 5. Linear range of the fluorimetric GALC assay.** (A) Recombinant human GALC enzyme was diluted in QCBP extract at a range of concentrations from 0 – 40 pg/μl. Data points represent the mean values of six replicates at each concentration with error bars showing the standard deviations from the mean. Graphpad analysis of the linear and second order polynomial fits show that linear fit was the preferred model. (B) Data points were collected for various incubation times up to a maximum of 20 h and represent the mean values of six replicates using a substrate concentration of 0.5 mM. The solid trend line shows that the activity is linear ($R^2=0.9819$) for the chosen incubation time of 17 hrs.
3.3.4. Analytical precision

The inter- and intra-day precision for each quality control sample are shown in Table 1. As outlined in the materials and methods section, three QC samples were analyzed for the single day and multi-day precision analyses. For the intra-day analyses, the percent of the coefficient of variation (CV) ranged from 5.2% to 6.7% for the low and high QC samples, respectively, whereas for the inter-day precision analyses, the %CV ranged from 8.8% to 14.4% for the low and high QC samples, respectively. For samples that are above the limit of quantitation (LoQ, described in the analytical sensitivity section), the highest intra-assay and inter-assay variability were 6.7% and 14.1% respectively (n=80). These variances are consistent with other assays utilized to monitor GALC activity in newborn screening settings [28].

3.4. GALC activity in newborn dried blood spots

To characterize the performance of our fluorimetric GALC assay, we analyzed DBS samples from presumed normal newborns (n = 396) and Krabbe patients (n=10), along with proficiency testing deficient (PT) spots (n=2). We found that the linear range of our fluorimetric GALC assay (established in Fig. 5) encompassed the entire range of samples tested. Fig. 6 shows that all patient and PT samples have very low enzyme activities with a narrow range (0.13–0.33 μmol/l/h for affected samples and 0.17–0.19 μmol/l/h for PT samples). The activity in the presumed normal samples shows a wider range (0.39–15.6 μmol/l/h) with a population mean of 2.108 μmol/l/h. As expected, GALC activity in the affected samples is significantly lower (p<0.0001 by one-way ANOVA) than in the presumed normal samples (0.25 ± 0.062 μmol/l/h for affected, 0.18 ± 0.01 μmol/l/h for PT, and 2.11 ± 1.47 μmol/l/h for normal). The mean values for all samples fall within the linear range of the assay, previously established in Fig. 5.

4. Discussion and conclusions

The results presented here establish a novel fluorimetric assay for measurement of GALC enzyme activity in dried blood spot specimens. The assay is pictorially depicted in Supplemental Fig. 1 and utilizes a novel substrate that is structurally improved from previous substrates for GALC [10,23] and has higher purity to minimize background fluorescence. A 3-carboxymethyl solubilizing group in the HMUG substrate yields the aglycone dye CHMU, which remains nonfluorescent as the CHMUG substrate. Hydrolysis of the glycosidic linkage between the sugar and the aglycone generates the fluorophore, which becomes fluorescent at high pH. Fluorescence assays for GALC activity have been previously described in fibroblasts [10] and leukocytes [11,12], which required laborious reagent preparation due to the hydrophobic nature of the HMUG substrate. Our novel substrate allows high throughput implementation due to the ease of reagent preparation. In combination with the optimized assay conditions (pH, substrate concentration, and high purity of the substrate), this novel substrate supports a robust method for determining GALC enzymatic activity (Figs. 2–5) that successfully discriminates normal DBS samples from samples that are deficient in GALC (Fig. 6).

GALC is a lysosomal enzyme that requires an acidic environment for full activity [15]. Under our assay conditions, GALC enzyme activity was 27.5% lower at pH 4.7 compared to the optimal pH of 4.3 (1.45 μmol/l/h at pH 4.7 vs 2.0 μmol/l/h at pH 4.3). Different assay types will require different conditions such as those run with mass spectrometry. Multiplexed tandem mass spectrometry (MS/MS) assays for GALC enzyme activity measurement use a single reaction buffer with common inhibitors, cofactors, and pH for multiple enzymes. In one such assay for a 6-plex MS/MS LSD protocol [13], a pH of 4.7 was used for all enzymes. The pH characterization studies presented herein demonstrate that peak GALC enzyme activity occurs at a very narrow range of pH: 4.0 to 4.3 (Fig. 2A).

Several recent publications [29–32] have suggested that calculations of analytical range, defined as the ratio of the QCH standard (typical of a non-affected newborn) divided by the blank [28], provide a reliable assessment of LSD enzyme assay performance. Application of this definition for analytical range using data presented in this study (QCH of 1.27 μmol/l/h (Table 1); Blank sample of 4 × 10⁻⁵ μmol/l/h (section 3.3.2 Analytical Sensitivity)) would result in an analytical range of 25,000 for our fluorimetric GALC assay, which is both absurd and a misrepresented analytical range. Reported values for other MS/MS lysosomal enzyme activity assays are orders of magnitude lower, for example, acid alpha-glucosidase (187 [29]), iduronate 2-sulfatase (243 [30]), or N-acetyl-alpha-glucosaminidase (580 [30]). According to CLSI’s Harmonized Terminology Database, “the measuring interval has been called the analytical measurement range, reportable range, analytical range, and calibration range” and it is truly defined as a “set of values of quantities of the same kind that can be measured by a given measuring instrument or measuring system with specified instrumental measurement uncertainty, under defined conditions.” [33]. It should be highlighted that without accounting for measurement uncertainty, calculation of analytical range is not a reliable method to assess assay performance. We report accepted analytical metrics such as LoB, LoD, and LoQ.

Table 1

| Sample | N | Mean | SD | CV | SD | CV |
|--------|---|------|----|----|----|----|
| QCBP   | 80| 0.109| 0.012| 11.1%| 0.022| 20.0%|
| QCL    | 80| 0.200| 0.010| 5.2%| 0.017| 8.8%|
| QCM    | 80| 0.842| 0.049| 5.8%| 0.066| 7.8%|
| QCH    | 80| 1.273| 0.086| 6.7%| 0.179| 14.1%|

SD – standard deviation, CV – coefficient of variation.
Our fluorimetric GALC assay protocol is significantly shorter than existing flow injection MS/MS GALC protocols [13] (~18 h for fluorimetry versus 34 h or longer for MS/MS including sample analysis). Additional benefits of our fluorimetric GALC assay include the use of standard laboratory equipment and a simple protocol with less than 30 min of hands-on effort and no laborious or caustic liquid extraction purification steps required.

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Declaration of competing interest

All the authors from Baebies have stocks or stock options in Baebies, Inc. Dr. Eckhardt was an employee of Advanced Liquid Logic, Inc. and is now an employee of Illumina, Inc. which acquired Advanced Liquid Logic, Inc.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plabm.2019.e00141.

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