Lymphocyte Galactocerebrosidase Activity by LC-MS/MS for Post-Newborn Screening Evaluation of Krabbe Disease

Hsuan-Chieh Liao,1,2 Zdenek Spacil,1,3 Farideh Ghomashchi,1 Maria L. Escolar,4 Joanne Kurtzberg,5 Joseph J. Orsini,6 Frantisek Turecek,1 C. Ronald Scott,7 and Michael H. Gelb1,8

BACKGROUND: Deficiency of the lysosomal enzyme galactosylcerebrosidase (GALC) causes Krabbe disease. Newborn screening for Krabbe disease is ongoing, but improved methods for follow-up analysis of screen-positive babies are needed to better advise families and to optimize treatment. We report a new assay for the enzymatic activity of GALC in lymphocytes.

METHODS: T lymphocytes were isolated from venous blood by magnetic bead technology. The assay used a close structural analog of the natural substrate and LC-MS/MS to quantify the amount of product with the aid of a chemically identical internal standard.

RESULTS: The analytical range of the assay (ratio of assay response for the QC high standard to that from all non–enzymatic-dependent processes) was 20-fold greater than that for the conventional radiometric GALC assay. The LC-MS/MS could distinguish cells that were null in GALC from those that contained traces of active enzyme (down to 0.3% of normal). There was a good correlation between the level of residual GALC activity in lymphocytes and the severity of Krabbe disease.

CONCLUSIONS: The new assay can measure small amounts of residual GALC activity in leukocytes with high accuracy compared to previous assays and can contribute, along with genotyping, biomarker analysis, and neurological imaging, a better plan for post–newborn screening follow-up for Krabbe disease.

© 2017 American Association for Clinical Chemistry

1 Department of Chemistry, University of Washington, Seattle, WA; 2 Institute of Clinical Medicine, National Yang-Ming University, Taipei, Taiwan; 3 Current affiliation: Masaryk University, Research Center for Toxic Compounds in the Environment, Kamence, Czech Republic; 4 Children’s Hospital of Pittsburgh, University of Pittsburgh Medical Center, Pittsburgh, PA; 5 Department of Pediatrics, Duke University, Durham, NC; 6 Division of Genetics, Wadsworth Center, New York State Department of Health, Albany, NY; Departments of 7 Pediatrics and 8 Biochemistry, University of Washington, Seattle, WA.

Received August 26, 2016; accepted March 20, 2017. Previously published online at DOI: 10.1373/clinchem.2016.264952
© 2017 American Association for Clinical Chemistry

Nonstandard abbreviations: GALC, galactocerebrosidase; MS/MS, tandem mass spectrometry; GALC-S, galactosylceramide with a 7-carbon heptanoyl chain; GALC-IS, GALC internal standard, GALC-P, GALC product; GLB1, human β-galactosidase-1; MRM, multiple reaction monitoring; LC, liquid chromatography; dpm, disintegration per minute; EIKD, early-infantile Krabbe disease; LOKD, late-onset Krabbe disease.
Results and Discussion

http://www.clinchem.org/content/vol63/issue8.

For reasons described below, we also tested the activity of recombinant human GALC because approximately 30% of the exons had been deleted from both copies of the galactosylceramidase (GALC) gene [homozygotes develop early infantile Krabbe disease (7)].

Methods

T lymphocytes were isolated from venous blood by magnetic bead technology. LC-MS/MS GALC assays were carried out with a close structural analog (galactosylceramide, with a heptanoyl fatty acyl chain) of the natural substrate. The internal standard is chemically identical to the substrate but differentiated by deuterium substitution. Standard operating procedures for all methods in this article are provided in the Supplemental Material file that accompanies the online version of this article at http://www.clinchem.org/content/vol63/issue8.

Methods

Statistical Analysis

The previously published buffer for in vitro assay of GALC was citrate-phosphate, pH 4.2 (6). We measured the activity of recombinant human GALC in this buffer and also in 0.2 mol/L sodium acetate, pH 4.0. Both buffers also contained sodium taurocholate and sodium olate, which activate GALC (6). For reasons described below, we preferred citrate phosphate buffer and used this for all subsequent studies.

LC-MS/MS ASSAY OF GALC

Before studies with blood cells we measured GALC in lysates of LCL (lymphoblastic cell line) lymphoblasts from the Coriell Cell Repository. The Pompe cell line GM13793 constituted our HIGH QC sample for the study (GM13793-HIGH). The second cell line, GM06805 (GM06805-LOW) was from a patient who was assumed to be completely devoid of functional GALC enzyme because approximately 30% of the exons had been deleted from both copies of the galactosylceramidase (GALC) gene [homozygotes develop early infantile Krabbe disease (7)].

Lysates from these cells were used to perform assays containing 2 μg of cell protein in an assay cocktail consisting of 150 μmol/L GALC-S and 5 μmol/L GALC-IS in citrate-phosphate buffer with sodium olate and sodium taurocholate, pH 4.2. The LC-MS/MS ion traces for GALC-P are shown in Fig. 1. Table 1 contains the ion counts for the GALC-P and GALC-IS obtained by integrating the multiple reaction monitoring (MRM) ion peaks vs the liquid chromatography (LC) retention time profile shown in Fig. 1. The complete assay, in which GM13793-HIGH lysate was incubated with GALC-S, is shown in the top panel of Fig. 1. The peak at approximately 1.67 min was due to GALC-generated GALC-P as it comigrated with the chemically identical GALC-IS (bottom panel). The peak in the top panel eluting at approximately 1.57 min is GALC-P produced by nonenzymatic cleavage of GALC-S in the heated electrospray ionization source of the MS/MS instrument as it comigrates with GALC-S (not shown). This insource cleavage
was of no consequence because the 2 GALC-P peaks were baseline separated and only the 1.67 min was integrated to determine the GALC activity.

The first control assay was the cell lysate (GM13793-HIGH) incubated without GALC-S (Fig. 1, panel 2), which showed essentially no GALC-P signal (0.007% of the activity seen with GM13793-HIGH in Fig. 1, panel 1, and Table 1). This indicated that the lysate did not contain compounds that were isobaric with GALC-P and that eluted at the same LC retention time. The second control was GALC-S incubated in buffer without cell lysate (Fig. 1, panel 4), which had a GALC-P signal that was only 0.07% of the signal seen with cell lysate (Table 1), showing that GALC-S contained essentially no GALC-P as an impurity and that GALC-S underwent essentially no nonenzymatic breakdown to GALC-P. Fig. 1, panel 3, shows the assay with GM06805-LOW incubated with GALC-S. The signal was approximately 10-fold higher than that obtained in the no-lysate and represented 0.4% of the signal seen with GM13793-HIGH (Table 1). This small increase in assay response was attributed to one or more non-GALC enzymes present in the cell lysate that were capable of generating GALC-P from GALC-S. One candidate is GLB1, since this enzyme cleaves terminal β-linked galactosyl residues from gangliosides. GLB1 deficiency causes 2 lysosomal storage disorders unrelated to Krabbe disease, namely GM1-gangliosidosis and Morquio B syndrome. For the assay with GM13793-HIGH the GALC-P assay response was 148 719 ion counts (mean of triplicate runs, Table 1), and thus 150-fold higher than the value seen with the GALC-null cells, GM06805-LOW.

![Fig. 1. LC-MS/MS ion chromatograms.](image)

**Fig. 1.** LC-MS/MS ion chromatograms.
Complete assay with GALC-S incubated with GM13793-HIGH cell lysate (panel 1); GM13793-HIGH lysate incubated in the absence of GALC-S (panel 2); GM06805-LOW lysate incubated with GALC-S (panel 3); and GALC-S incubated in buffer without cell lysate (panel 4).
Panels 1–4 show the MRM ion chromatograms for GALC-P; panel 5 is for GALC-IS.

### Table 1. LC-MS/MS GALC assay results.

<table>
<thead>
<tr>
<th>Assay</th>
<th>GALC-P ion counts</th>
<th>GALC-IS ion counts</th>
<th>GALC activity, nmol/h/mg, mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM13793-HIGH lysate + GALC-S</td>
<td>143 518</td>
<td>124 6449</td>
<td>0.5837 ± 0.0406</td>
</tr>
<tr>
<td></td>
<td>147 838</td>
<td>117 1068</td>
<td></td>
</tr>
<tr>
<td></td>
<td>154 801</td>
<td>117 1068</td>
<td></td>
</tr>
<tr>
<td>No-lysate control</td>
<td>89</td>
<td>123 1481</td>
<td>0.000364 ± 0.000119</td>
</tr>
<tr>
<td></td>
<td>64</td>
<td>115 0753</td>
<td></td>
</tr>
<tr>
<td></td>
<td>122</td>
<td>115 6488</td>
<td></td>
</tr>
<tr>
<td>GM13793-HIGH lysate minus GALC-S</td>
<td>10</td>
<td>563 384</td>
<td>7.25 × 10⁻⁵ ± 0.979 × 10⁻⁵</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>586 144</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>532 992</td>
<td></td>
</tr>
<tr>
<td>GM06805-LOW + GALC-S</td>
<td>1129</td>
<td>135 2893</td>
<td>0.00371 ± 0.00038</td>
</tr>
<tr>
<td></td>
<td>1110</td>
<td>131 6255</td>
<td></td>
</tr>
<tr>
<td></td>
<td>880</td>
<td>126 0690</td>
<td></td>
</tr>
</tbody>
</table>

* Instrument noise was measured to be 16 based on integration of the noise region adjacent to the analyte peak over the same time window as for the peak integration.
We explored a number of previously reported glyco-
yhydrolase inhibitors to see if the assay response in
GM06805-LOW cells could be reduced toward the value
seen in the no-lysate control. We also tested the inhibitor
on GM13793-HIGH cells to explore the effect of the
compound on GALC. The results, summarized in the
online Supplemental Material file, suggested that the re-
sidual activity in GM06805-LOW cells was due to one or
more enzymes other than GALC, possibly GBL1. The
results also showed that the buffer composition had a
large effect on the substrate specificity of enzymes in the
cell lysates capable of cleaving GALC-S.

ANALYTICAL RANGE AND ACCURACY OF DETECTION OF
SMALL AMOUNTS OF RESIDUAL GALC ACTIVITY
We defined the analytical range as the enzyme-dependent
assay response observed with the HIGH quality control
sample (GM13793-HIGH, typical of the activity in cells
from a healthy individual) divided by the assay response
from all enzyme-independent processes. The analytical
range for the LC-MS/MS, discussed in detail in the on-
line Supplemental Material file, had a value of 149. As
shown in the online Supplemental Material file, the larg-
est contribution to the enzyme-independent assay re-
sponse was conversion of GALC-S to GALC-P by one or
more non-GALC components of the cell lysate.

Since the analytical range for the LC-MS/MS
GALC assay was larger than that for previously reported
GALC assays (see below), we tested the possibility of
detecting a difference in assay response due to very
small changes in the amount of GALC in cell lysates.
To study this, we added small amounts of GALC-
containing lysate (GM13793-HIGH) to GALC-null
cell lysate (GM06805-LOW) so that the total amount
of cellular protein was the same in all assays (2 or 5
μg). The results are shown in Fig. 2. Assays were con-
ducted in triplicate, and the mean assay response was
obtained along with the estimated error. The LC-
MS/MS ion chromatograms are shown in online Supple-
mental Fig. 2. The data showed that very small differ-
ences in GALC enzymatic activity at the low end could be
observed in a statistically significant way. For example,
the ratio of GALC-P to GALC-IS ion counts when
100% GM06805-LOW lysate was used was 0.0012
(0.000044), and it was 0.00014 (0.000015) for the no-
lysate blank. The difference in these values was 0.0011
(0.000046) and was thus of high confidence. This differ-
ence was 8-fold the value for the no-lysate control. The
GALC-P/GALC-IS ion count ratio observed with 100%
GM13793-HIGH lysate was 0.2999, thus the 100%
GM06805-LOW lysate produced 0.4% of the GALC
activity as the GM13793-HIGH lysate. Under our assay
conditions, this was the contribution by one or more
non-GALC enzymes in the lysate (possibly GLB1).

As shown in Fig. 2 (right), when 5 μg of cell lysate
protein was used per assay, an increase in GALC activity
above that measured with 100% null lysate (GM06805-
LOW) was readily measured when this lysate was spiked

![Fig. 2](image-url)
with 0.5% GM13793-HIGH lysate. The activity increased linearly as the fraction of GM13793 was increased.

We believe that it will be clinically useful to compare this discrimination between small differences in GALC activity to what was feasible with the previously developed assay of GALC, which was used to support follow-up studies of Krabbe disease newborn screening–positive patients (9). The previous GALC assay made use of radiolabeled galactosyleceramide that contained tritium attached to C6 of the galactosyl group (5). Using the specific radioactivity of the tritiated galactosylceramide substrate, the micrograms of peripheral blood mononuclear cell (mainly lymphocytes) protein per assay, and the reported GALC activity (5), approximately 400 disintegrations per minute (dpm) of tritium were detected using this radiometric assay. We have carried out this radiometric assay and observed an assay response of 406 and 433 dpm for duplicate runs using 50 μg of cell lysate protein per assay (see online Supplemental Material) (5). This number agrees with the published value (5). In a control assay with omission of cell lysate, we observed assay responses of 49 and 53 dpm for duplicate runs (see online Supplemental Material). Thus, the analytical range for this assay was (420 – 50)/50 = 7.4, which was 20-fold less than that for the LC-MS/MS GALC assay. As noted above, an increment in GALC activity corresponding to 0.4% of that measured with GM13793-HIGH was observed with the LC-MS/MS with high confidence. This corresponded in the radiometric GALC assay to 0.004 × (420 – 50) = 1.5 dpm, which was only 3% of the dpm value measured in the no-lysate control. When we carried out the no-lysate control as 5 replicates we obtained a mean (SD) blank value of 49 (3) dpm. Clearly it was not possible to measure increments of GALC activity with the radiometric assay of <2%–3% of normal activity.

Also of note, the radiometric assay was performed with 50 μg of cell lysate protein and required 2–3 mL of patient blood, whereas the LC-MS/MS assay was performed with 10- to 20-fold less protein and 0.5 mL of patient blood was sufficient for >15 GALC assays, thus allowing triplicate assays to be routinely performed. It is difficult to obtain >2–3 mL of blood from newborns.

### Table 2. GALC activities measured by LC-MS/MS in T-lymphocytes isolated from patient blood.*

<table>
<thead>
<tr>
<th>Patient</th>
<th>Approximate age of onset of symptoms</th>
<th>Age at time of blood draw</th>
<th>GALC activity (% of GM13763-HIGH), mean ± SD*</th>
<th>Psychosine in blood, nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>EIKD-1</td>
<td>&lt;1 month</td>
<td>&lt;1 month</td>
<td>0.49 ± 0.09 (0.42 ± 0.08)b</td>
<td>15.7</td>
</tr>
<tr>
<td>EIKD-2</td>
<td>&lt;1 month</td>
<td>&lt;1 month</td>
<td>0.35 ± 0.02 (0.38 ± 0.04)b</td>
<td>26.1</td>
</tr>
<tr>
<td>EIKD-3</td>
<td>&lt;1 month</td>
<td>&lt;1 month</td>
<td>0.30 ± 0.08 (0.29 ± 0.08)b</td>
<td>20.4</td>
</tr>
<tr>
<td>LOKD-1 (late infantile)</td>
<td>10 years</td>
<td>12 years</td>
<td>1.72 ± 0.31 (1.79 ± 0.28)b</td>
<td>6.8</td>
</tr>
<tr>
<td>High Risk-KD-1</td>
<td>asymptomatic, high risk</td>
<td>&lt;1 month</td>
<td>1.46 ± 0.17</td>
<td>3.0</td>
</tr>
<tr>
<td>Moderate Risk-KD-1</td>
<td>asymptomatic, moderate risk</td>
<td>&lt;1 month</td>
<td>11.02 ± 1.28</td>
<td>0.4</td>
</tr>
<tr>
<td>Moderate Risk-KD-2</td>
<td>asymptomatic, moderate risk</td>
<td>&lt;1 month</td>
<td>5.78 ± 0.24</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*EIKD has onset of symptoms at <1 month. High Risk-KD and Moderate Risk-KD patients are asymptomatic newborns (see main text for description of risk category). Error bars are the SDs from triplicate analyses.

Numbers in parentheses are repeat triplicate analyses done after 4 months of storage at −80 °C of the lysates assayed originally.

High-Accuracy GALC MS/MS
imately 20 repeats of our Quality Control High (GM13763-HIGH lymphoblasts) carried out over an approximately 1-year period was 6%.

All 3 early infantile Krabbe disease patients showed GALC activity in T lymphocytes that was the same as that seen in GM06805-LOW LCL lymphoblasts, which are null for GALC. On the other hand, the late-onset Krabbe and the asymptomatic newborn patients had statistically significant higher levels of GALC. The number of patients analyzed was too small to make a firm conclusion that the new GALC assay can better predict the onset of Krabbe disease.

Table 3 shows the radiometric GALC activity in lymphocytes for 14 patients previously identified by the New York Krabbe disease newborn screening program. All 14 patients were placed in the high-risk category by the newborn screening program based on leukocyte radiometric GALC of H113490.15 nmol product/h/mg protein (Table 3). In this table genotypes are given along with Krabbe disease status. Four patients were confirmed by clinical examination to have early infantile Krabbe disease. The remaining 10 patients have not displayed Krabbe disease symptoms as far as we know. All 4 patients with early infantile Krabbe disease had 2 severe mutations in both copies of the GALC gene (either the 30-kb deletion or a frame shift or a C-terminal extension of 42 amino acids). There was no general trend that the radiometric GALC assay gave lower enzymatic activity for symptomatic vs asymptomatic patients. In fact, 1 of the symptomatic patients (EIKD-4) displayed a GALC activity of 0.12 nmol/h/mg protein, which was 12-fold higher than the activity measured in EIKD-1 and was as high or higher than GALC activity in all of the asymptomatic patients. EIKD-4 is homozygous for the 30-kb deletion and should be devoid of functional GALC protein. Thus, the radiometric GALC assay gave activity values that were not correlated with strong predictions based on severe genotypes or with the severity of Krabbe disease. Estimated errors in the values in Table 3 were not available.

STABILITY OF GALC IN WHOLE BLOOD
Freshly drawn blood must be shipped to the biochemical genetics laboratory where the GALC assay will be performed. It was thus important to study the stability of GALC in whole blood before isolation of T lymphocytes. Whole blood was collected into a K2EDTA collection tube, and 0.5-mL aliquots were stored at ambient temperature (approximately 21 °C) or at 4 °C for up to 5 days. At regular intervals, T lymphocytes were isolated and submitted to LC-MS/MS assay of GALC enzymatic activity. Blood stored up to 3 days at 4 °C lost ≤15% of the initial GALC activity, whereas blood stored at ambient temperature for up to 3 days lost up to 30% of the initial GALC activity (Table 4). On the basis of these results, we recommend that blood be collected, stored at 4 °C until shipment the same day, and sent for arrival in <3 days with frozen gel packs at approximately 4 °C.

PSYCHOSINE ANALYSIS IN DRIED BLOOD SPOTS FROM KRABBE PATIENTS
We also measured the concentration of psychosine (galactosylsphingosine) in dried blood spots from the patients listed in Table 2. Psychosine may be a physiologi-

<table>
<thead>
<tr>
<th>Patient</th>
<th>Radiometric GALC activity, nmol/h/mg protein</th>
<th>Genotype</th>
<th>Krabbe disease status</th>
</tr>
</thead>
<tbody>
<tr>
<td>EIKD-4</td>
<td>0.01</td>
<td>p.R168C g.30Kb Del // p.R168C g.30Kb Del</td>
<td>Infantile Krabbe disease</td>
</tr>
<tr>
<td>EIKD-5</td>
<td>0.05</td>
<td>p.R168C g.30Kb Del // p.I546T g.30Kb Del</td>
<td>Infantile Krabbe disease</td>
</tr>
<tr>
<td>EIKD-6</td>
<td>0.02</td>
<td>p.R168C g.30Kb Del // p.R168C g.30Kb Del</td>
<td>Infantile Krabbe disease</td>
</tr>
<tr>
<td>EIKD-7</td>
<td>0.12</td>
<td>p.R168C g.30Kb Del // p.G360DfsX2</td>
<td>Infantile Krabbe disease</td>
</tr>
<tr>
<td>High Risk-KD-2</td>
<td>0.06</td>
<td></td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>High Risk-KD-3</td>
<td>0.12</td>
<td></td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>High Risk-KD-4</td>
<td>0.07</td>
<td></td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>High Risk-KD-5</td>
<td>0.09</td>
<td></td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>High Risk-KD-6</td>
<td>0.12</td>
<td></td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>High Risk-KD-7</td>
<td>0.03</td>
<td></td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>High Risk-KD-8</td>
<td>0.05</td>
<td></td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>High Risk-KD-9</td>
<td>0.05</td>
<td></td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>High Risk-KD-10</td>
<td>0.06</td>
<td></td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>High Risk-KD-11</td>
<td>0.07</td>
<td></td>
<td>Asymptomatic</td>
</tr>
</tbody>
</table>
Table 4 Whole blood GALC stability studies.

<table>
<thead>
<tr>
<th>Blood storage</th>
<th>GALC activity, nmol/h/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh blood (adult 1)</td>
<td>3.66</td>
</tr>
<tr>
<td>3 Days at 4 °C storage (adult 1)</td>
<td>3.91</td>
</tr>
<tr>
<td>Fresh blood (adult 2)</td>
<td>3.72</td>
</tr>
<tr>
<td>1 Day at ambient temperature (adult 2)</td>
<td>2.18</td>
</tr>
<tr>
<td>2 Days at ambient temperature (adult 2)</td>
<td>2.10</td>
</tr>
<tr>
<td>3 Days at ambient temperature (adult 2)</td>
<td>2.84</td>
</tr>
<tr>
<td>Fresh blood (adult 3)</td>
<td>3.55</td>
</tr>
<tr>
<td>1 Day at 4 °C (adult 3)</td>
<td>3.02</td>
</tr>
<tr>
<td>2 Days at 4 °C (adult 3)</td>
<td>3.15</td>
</tr>
<tr>
<td>3 Days at 4 °C (adult 3)</td>
<td>3.25</td>
</tr>
</tbody>
</table>

...tent; thus, it was critical to be able to measure GALC enzymatic activity very accurately in the 0%–5% range. The LC-MS/MS method developed in this study showed the analytical resolution to accomplish this. Further studies may prove whether measurement of psychosine or accurate GALC activity or both is best at predicting the severity of Krabbe disease.

PROSPECTS FOR KRABBE DISEASE DIAGNOSIS AND PREDICTION

The main purpose of this report was to show that it was possible to develop an assay of GALC that had a much higher accuracy than the currently used radiometric GALC assay. The preliminary data on a small number of patients supports the new LC-MS/MS GALC as having the potential to predict which patients will develop Krabbe disease early in life, later on, or not at all.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures or Potential Conflicts of Interest: Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Research Funding: M. Escolar, Legacy of Angels Foundation; M.H. Gelb, NIH (R01 DK067859) and Legacy of Angels Foundation Grant (www.loanf.org).
Expert Testimony: None declared.
Patents: None declared.
Role of Sponsor: The funding organizations played a direct role in the design of study, choice of enrolled patients, review and interpretation of data, preparation of manuscript, and final approval of manuscript.

References