Tandem Mass Spectrometry for the Direct Assay of Enzymes in Dried Blood Spots: Application to Newborn Screening for Krabbe Disease, *Yijun Li,[1] Knut Brockmann,[2] Frantisek Turecek,[1] C. Ronald Scott,[3] and Michael H. Gelb[1,4*]* (Departments of [1] Chemistry, [3] Pediatrics, and [4] Biochemistry, University of Washington, Seattle, WA; [2] Department of Pediatrics and Neuropediatrics, Children's Hospital, University of Goettingen, Goettingen, Germany; * address correspondence to this author at: Departments of Chemistry and Biochemistry, University of Washington, Campus Box 351700, Seattle, WA 98195; fax 206-685-8665, e-mail gelb@chem.washington.edu)

Tandem mass spectrometry (MS/MS) for newborn screening allows detection of abnormal or excess metabolites in dried blood spots *(1–5)*. Dried blood spots provide a source of active enzymes for the detection of Fabry *(6)*, Hunter *(7)*, Hurler *(7)*, Pompe *(8)*, Gaucher *(9)*, Niemann–Pick *(9)*, and Tay–Sachs *(10)* diseases. We investigated whether MS/MS can be used to directly assay enzymes in dried blood spots, in this case, galactocerebroside β-galactosidase (GALC; EC 3.2.1.46) for the detection of Krabbe disease. Development of a GALC assay is challenging because of the low GALC activity in cell lysates. It has been argued that a neonatal screening method for Krabbe disease is needed *(11)*. This is the first report of an assay for Krabbe disease that uses dried blood spots and the first use of MS/MS for direct enzyme assay from newborn-screening cards.

The GALC assay uses commercially available reagents and is based on the reaction shown in Fig. 1 and the use of electrospray ionization (ESI) MS/MS. GALC converts β-Gal-C_8-Cer to C_8-Cer. A known amount of substrate analog with two extra methylene groups, C_{10}-Cer, is added as internal standard. After collision-induced dissociation, C_8-Cer and C_{10}-Cer give rise to the same fragment ion (*m/z* 264.3); thus C_8-Cer and C_{10}-Cer are distinguished by the Q1 quadrupole. The indicated fragmentation pathway is the major process, allowing for high-sensitivity detection of C_8-Cer and C_{10}-Cer.

A simple, one-step, solid-phase extraction procedure was developed (see below) to remove the large amount of detergent before ESI-MS/MS. The product C_8-Cer and internal standard C_{10}-Cer have nearly identical ESI-MS/MS ionization efficiencies (see Fig. 1 in the online Data Supplement that accompanies this Technical Brief at http://www.clinchem.org/content/vol50/issue3/). Studies with a second internal standard, C_{12}-Cer, showed essentially no loss of C_8-Cer product and C_{10}-Cer internal standard during sample workup and the assay incubation period.

Dried blood spots were obtained in compliance with Institutional Review Board review, were stored at −20 °C (no longer than 10 days at room temperature during transport) in zip-lock plastic bags (three layers), and were analyzed within 70 days. The GALC assay mixture was prepared as follows. D-Galactosyl-β1-1′-ceramide (C8:0; β-Gal-C_8-Cer; Avanti) substrate stock solution (200 μL of 3 mmol/L solution in methanol) and ceramide (C10:0; C_{10}-Cer; Avanti) internal standard stock solution (240 μL of 0.05 mmol/L solution in methanol) were added to a 5-mL glass vial, and the solvent was removed in a vacuum desiccator. We then added 240 μL of an aqueous solution of sodium taurocholate (120 g/L) and oleic acid (12 g/L) to the vial, vortex-mixed the sample until the residue dissolved, and then added 2.76 mL of citrate–phosphate buffer, pH 4.5 (0.2 mol/L Na_2HPO_4, 0.1 mol/L sodium citrate; pH adjusted with 6 mol/L HCl). The vial was vortex-mixed, and any emulsion was broken up by centrifugation. The stock assay solution (3.0 mL) contained 0.2 mmol/L substrate, 4.0 μmol/L internal standard, 9.6 g/L sodium taurocholate, 0.96 g/L oleic acid, and citrate–phosphate, pH 4.5 and was stored at −20 °C (multiple freeze–thaw cycles tolerated).

To each 1.7-mL Eppendorf tube we added a 2-mm diameter dried blood spot followed by 25 μL of GALC assay mixture. Tubes were briefly centrifuged at 5000*g* to pellet the liquid. Tubes were incubated for 20–24 h at 37 °C with orbital shaking (150 rpm) in an air shaker, after which 400 μL of methanol–$CHCl_3$ (2:1 by volume) was added. The liquid was aspirated up and down five times with a Pipetman P1000, and 350 μL of $CHCl_3$ and 350 μL of H_2O were added. The tube was centrifuged at 5000*g* for 2 min, after which the bottom layer (300 μL) was removed

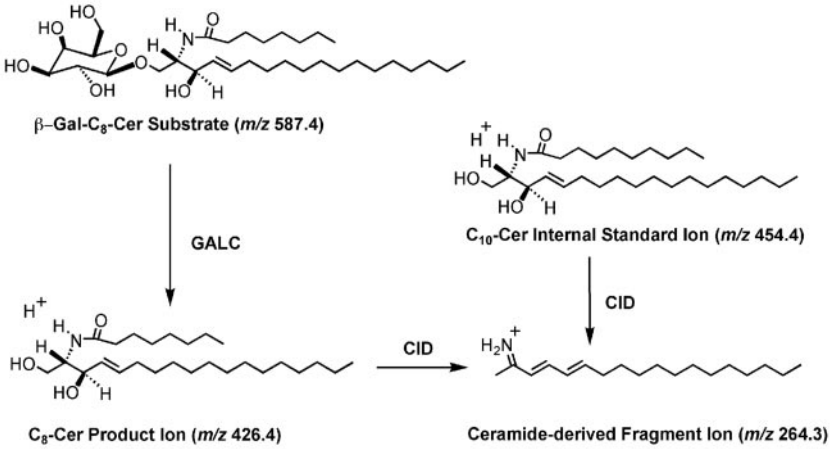

Fig. 1. Reaction scheme for the assay of GALC by ESI-MS/MS.

Shown is the GALC reaction, the monoprotonated ions derived from the GALC reaction product (C_8-Cer) and internal standard (C_{10}-Cer), and the dominant collision-induced dissociation (*CID*) of these ions to give the common ion of *m/z* 264.3, which is detected by ESI-MS/MS with a triple-quadrupole instrument.

with a Pipetman P1000 and transferred to a glass-wool-plugged 1-mL Pipetman tip that was filled with silica gel (90–110 mg; 230–400 mesh; Merck). The pipet tip was placed in a glass tube, and solvent was pushed through the silica gel for ~2 s with compressed air. Methanol–$CHCl_3$ (600 μL; 1:9 by volume) was applied to the silica gel and pushed through with compressed air. The solvent was removed in a vacuum desiccator, and the residue was suspended in 200 μL of 5 mmol/L ammonium formate in methanol–$CHCl_3$ (3:1 by volume) for ESI-MS/MS.

ESI-MS/MS was performed on a Sciex API-III Plus tandem quadrupole instrument operating in positive-ion, parent-ion scan mode with the following settings: needle voltage (ISV), 4300 V; counter electrode (IN), 650 V; nebulizer gas, 35 psi; curtain gas, 1.2 L/min; skimmer voltage (OR), 45 V; quadrupole d.c. offset (R0), 30 V; quadrupole 1 resolution (RE1), 110; quadrupole 3 resolution (RE3), 105; collision gas number density (CGT), $\sim 150 \times 10^{12}$ molecules/cm^3; Q2 collision gas, argon; parent ion, *m/z* 264.0; dwell time, 3.0 ms; pause time, 0.052 s; step size, 0.2 atomic mass units (amu); Q1 scan range (C_8-Cer), *m/z* 425.4–428.4; scan range (C_{10}-Cer), *m/z* 453.4–456.4. A sum of 200 scans over ~0.3 min was used for quantification. Samples were introduced by infusion at 3.3 μL/min through a fused-silica capillary (100 μm i.d.) with a syringe drive. The capillary was flushed with acetonitrile (4 times 50 μL) after each sample. The amount of product was calculated from the ion abundance ratio of product (C_8-Cer) to internal standard (C_{10}-Cer) for a sample minus that of a blank, multiplied by the amount of added internal standard and divided by the response ratio of product to internal standard.

ESI-MS/MS parent ion scans for the GALC assay performed with a single 2-mm dried blood spot as well as a control assay containing a 2-mm blood-free paper disk are shown in Fig. 2 in the online Data Supplement. The product-to-internal standard mole ratio was typically approximately eightfold greater in the blood-containing sample than in the control. Assay optimization showed maximum GALC activity at pH 4.5 and ~9 g/L sodium taurocholate (Fig. 3 in the online Data Supplement). The amount of C_8-Cer product increased linearly with the incubation time from 0 to 24 h, and the reaction velocity showed saturation kinetics with variation of the substrate concentration [K_m = 0.24 mmol/L; V_{max} = 4.0 $\mu mol \cdot h^{-1} \cdot (L\ blood)^{-1}$; Fig. 4 in the online Data Supplement].

We carried out 14 assays using 2-mm blood spots from the same individual. The CV for the amount of product after 24 h was 9.6%. We submitted the extract from a single assay to 17 ESI-MS/MS measurements over a 2-month period (extract was stored at room temperature); the CV was 5.6%. Thus the GALC assay is highly precise, and the MS signals for C_8-Cer and C_{10}-Cer are highly reproducible. When dried blood spots from four individuals were stored at −20 °C or room temperature for 70 days, the GALC activity decreased by a mean (SD) of 9.6 (3.8)% and 40.5 (5.1)%, respectively. Storage of dried blood spots at room temperature for 10 days led to a 9.5 (3.9)% decrease in GALC activity (data obtained from dried blood spots from two healthy individuals and two patients with Krabbe disease). Thus, GALC activity in dried blood spots is sufficiently stable to permit transport of samples at ambient temperature.

We measured the GALC enzyme activity in dried blood spots from 16 healthy individuals and from 5 individuals who were previously diagnosed with Krabbe disease. We also carried out GALC assays on samples from four patients with Fabry disease and three with Gaucher disease. The results are summarized in Table 1. Activity among healthy individuals ranged from 1.4 to 3.7 $\mu mol \cdot h^{-1} \cdot (L\ blood)^{-1}$, whereas activity for Krabbe patients was much lower [0.05–0.23 $\mu mol \cdot h^{-1} \cdot (L\ blood)^{-1}$]. For the patients with Fabry and Gaucher disease, the GALC assay gave values that are typical of those obtained for healthy individuals, although a larger range of specific activity values was observed (Table 1). These results suggest that an assay for GALC in dried blood spots from newborns can be implemented and evaluated for the early detection of Krabbe disease.

The GALC assay most often reported in the literature is based on a radiometric assay *(12)*. The ESI-MS/MS assay avoids the use of radioactive material, which may not be practical for high-throughput newborn screening. Measurement of GALC activity with a series of substrates that differed in the lengths of their fatty acyl groups revealed that C8:0 ceramide gives fivefold higher activity than substrates containing ceramides with the longer chain fatty acids found in natural ceramides *(12)*, a factor that was important in our ability to detect GALC in dried blood spots. The use of β-Gal-C_8-Cer also has an advantage in that it generates the unnatural product C_8-Cer, thus avoiding interference from the natural ceramides present in biological specimens.

Table 1. GALC activities measured in 2-mm dried blood spots.

Group	No. of samples	Individual GALC activities,[a] $\mu mol \cdot h^{-1} \cdot (L\ blood)^{-1}$	Range of GALC activities, $\mu mol \cdot h^{-1} \cdot (L\ blood)^{-1}$
Healthy individuals	16	1.46, 1.43, 1.64, 1.59, 1.65, 1.61, 1.88, 1.97, 1.79, 2.15, 1.94, 2.78, 2.23, 3.24, 3.75	1.43–3.75
Fabry patients	4	2.00, 1.28, 5.34, 1.57	1.28–5.34
Gaucher patients	3	1.35, 1.76, 7.49	1.35–7.49
Krabbe patients	5	0.08, 0.05, 0.23, 0.07, 0.08	0.05–0.23

[a] The mean GALC activity [0.30 $\mu mol \cdot h^{-1} \cdot (L\ blood)^{-1}$] measured in 2-mm blood-free paper disks [values from four determinations were 0.27, 0.31, 0.31, and 0.31 $\mu mol \cdot h^{-1} \cdot (L\ blood)^{-1}$] has been subtracted from the observed GALC activities to give the values listed in the table. All GALC activities were calculated assuming that a 2-mm paper disk contains 1.27 μL of blood *(13)*.

Because the enzymatic products are resolved on the basis of their m/z ratios, we are exploring the use of MS/MS for the quantification of multiple enzymes in a single dried blood spot by use of a single-platform analytical method in much the same way that MS/MS is currently used to determine the concentrations of multiple metabolites in a single dried blood spot.

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Comprehensive Mu-Opioid-Receptor Genotyping by Pyrosequencing, *Carsten Skarke,* Anja Kirchhof, Gerd Geisslinger, and Jörn Lötsch* (*pharmazentrum frankfurt*, Institute of Clinical Pharmacology, Johann Wolfgang Goethe-University, Theodor Stern Kai 7, 60590 Frankfurt, Germany; * author for correspondence: fax 49-69-6301-7636, e-mail skarke@em.uni-frankfurt.de)

The human μ-opioid receptor, encoded by the *OPRM1* gene *(1, 2)*, is the major site for the analgesic action of opioids. The *OPRM1* gene is therefore a first-line candidate for evaluating the role of mutations on the clinical effects of opioids. The mutant allele of the 118A>G single-nucleotide polymorphism (SNP) in the *OPRM1* gene, which codes for an Asn40Asp μ-opioid receptor, has been associated with decreased opioid activity in carriers of the 118G allele. Morphine 6-glucuronide (M6G) and morphine have lower potencies for pupil constriction in carriers of the mutation, who also vomit less often after treatment with M6G than noncarriers *(3)*. Carriers of the 118G allele need more alfentanil for postoperative analgesia but have less pain relief than noncarriers *(4)*. The 118G allele has also been associated with a greater cortisol response to opioid receptor blockade with naloxone *(5)*.

Regarding opioid addiction, the mutant allele of the 17C>T SNP was found more frequently in drug addicts than in nonaddicts *(6, 7)*. An association between the mutant alleles of the 118A>G (exon 1) and 691C>G (intron 2) SNPs and opioid dependence was reported for Chinese heroin addicts, although this was based on a small study group *(8)*. The frequency of the mutated 118G allele was higher in Indian heroin addicts than in controls *(9)*. Addicted individuals carrying both the mutated 118G allele and the mutated 31A allele in intron 2 consumed higher doses of heroin than individuals who did not carry these mutations *(10)*. The simultaneous presence of the mutated alleles for SNPs −1793T>A, −1699-(−1698)insT, −1320A>G, −111C>T, and 17C>T is associated with substance dependence *(11)*. In European Americans, allele −2044A and haplotypes that include −2044A were found to be associated with susceptibility for substance dependence *(12)*.

To promote further investigation of an association of *OPRM1* mutations with altered opioid effects or substance dependence, we describe a rapid screening method for several mutations in the *OPRM1* gene. SNPs in the *OPRM1* gene that qualify for large-scale screening in patients were selected to become part of the screening method when they met one of the following three criteria: (*a*) in vitro or human studies had revealed a functional consequence; (*b*) the mutation causes an amino acid exchange, encoding an altered opioid receptor protein; or (*c*) the SNP has a high reported allelic frequency, which implies that it could have immediate clinical relevance for the administration of opioids in a large part of the population. On that basis, a total of 23 SNPs in the promoter region; in exons 1, 2, and 3; and in the second intron were chosen for screening (see Table 1 for details). To this we added the SNPs −54G>T in the promoter, 24G>A in exon 1 and 942G>A in exon 3 because their close proximity to SNPs −38C>A, 17C>T and 877G>A, respectively, allowed their detection by use of the already available PCR templates with a small extension of the respective assays (see the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue3/). Thus, our method includes a total of 26 SNPs.

To detect the 26 selected SNPs in *OPRM1*, we developed a set of 14 assays (Table 1) based on the real-time pyrophosphate detection method Pyrosequencing™ *(13)*.