Published in final edited form as:

Mol Genet Metab. 2011 January ; 102(1): 33–40. doi:10.1016/j.ymgme.2010.08.018.

Ultra Fast and Sensitive Liquid Chromatography Tandem Mass Spectrometry Based Assay for Galactose-1-Phosphate Uridyltransferase and Galactokinase Deficiencies

Yijun Li¹, Adam S. Ptolemy², Lauren Harmonay¹, Mark Kellogg², and Gerard T. Berry^{*,1}

¹The Manton Center for Orphan Disease Research, Division of Genetics, Department of Pediatrics

²Department of Laboratory Medicine, Children's Hospital Boston, Harvard Medical School, Boston, MA, USA

Abstract

The diagnosis of transferase and galactokinase deficiency galactosemia usually involves the measurement of erythrocyte galactose-1-phosphate uridylyltransferase (GALT) and galactokinase (GALK) enzyme activity, respectively. The current gold standard assays for these enzymes are radioactive assays, which are laborious and/or incapable of measuring low enzyme activities. To further our knowledge of genotype-phenotype relationships, we had developed an assay for GALT activity alone using LC-MS/MS. In this study we generated a robust and sensitive LC-MS/MS based GALT and GALK assay using a novel normal phase chromatographic condition. We improved upon our earlier assay by drastically reducing the instrument run time and eliminating the use of an ion pairing reagent. Stable isotope labeled substrates were utilized in the GALT and GALK assays. The enzymatic products ($[^{13}\text{C}_6]$ -uridine diphosphate galactose in GALT assay and $[^{13}\text{C}_6]$ -galactose-1-phosphate in GALK assay) were quantified in a 3 min LC-MS/MS run. The assays were sensitive enough to allow for the quantification of enzyme activities as low as 0.2% and 0.3% of normal control values in the GALT and GALK assays, respectively. Thirty-three samples from non-galactosemic patients were assayed to have erythrocyte GALT activity of 23.4 ± 4.2 and GALK activity of 1.8 ± 0.47 (mean $\pm$ SD) $\mu\text{mol} \cdot (\text{g Hgb})^{-1} \cdot \text{hr}^{-1}$. Erythrocyte GALT activities in a cohort of 16 patients with classic galactosemia were measured: 4 patients had GALT activity less than 1% of normal control values and the remaining 12 had no detectable GALT activity. No GALK activity was detected in a GALK deficient sample we analyzed. Lastly, we tested the feasibility of adapting this LC-MS/MS based GALT/GALK assay as a newborn screening (NBS) test.

Keywords

Galactosemia; galactokinase (GALK); galactose-1-phosphate uridylyltransferase (GALT); Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Introduction

Classic galactosemia caused by GALT (EC 2.7.7.12) deficiency was one of few inborn errors of metabolism included in the earliest newborn screening program in the USA.

* Address correspondence to: GT Berry, Children's Hospital Boston, 300 Longwood Ave, Boston, MA 02115 United States. Tel: (617) 355-4316, Fax: (617) -730-0788, gerard.berry@childrens.harvard.edu.

Human gene: GALT gene, galactose-1-phosphate uridylyltransferase gene.

Newborns of all 50 American states are currently being screened for this disorder, with its frequency estimated to be 1/30,000 to 1/60,000 live births in the USA and in other multi-ethnic populations [1]. Patients without a timely diagnosis and treatment usually present with jaundice, liver disease, anemia, encephalopathy, cataracts, and succumb to death in early infancy due to sepsis. Early diagnosis and treatment by a lactose restricted diet is crucial to prevent death and alleviate these symptoms [2, 3, 4]. The diagnosis of classic galactosemia usually involves the measurement of erythrocyte GALT activity. The current GALT enzyme assays in many diagnostic laboratories are radioactive assays, in which C-14 radioactive labeled galactose-1-phosphate is used as the enzyme substrate and the C-14 radioactive labeled enzymatic product, uridine diphosphate galactose (UDPGal), is separated from the substrate by either paper or by ion-exchange chromatography prior to detection. The radioactivities in either the different segments of the paper chromatography or eluent fractions of the ion-exchange chromatography are then measured by scintillation counting to determine the amount of enzymatic product. This assay is not only labor intensive and time consuming, but also unreliable when GALT activity is low, in part because of a purity issue with the radioactive substrate. It was noticed that the C14- galactose-1-phosphate (Gal-1-P) often contained trace amount of C-14 labeled glucose-1-phosphate (Glu-1-P), which would lead to the formation of C14-labeled uridine diphosphate glucose (UDPGlc) that is not distinguishable from the enzymatic product (C14-UDPGal) and results in artificially elevated enzymatic activity [5].

GALK (EC 2.7.1.6) and uridine diphosphate galactose 4'-epimerase (GALE, EC 5.1.3.2) deficiencies are the two other known causes of galactosemia. The main clinical presentation in patients with GALK deficiency is cataract caused by the accumulation of galactitol in the lens and it is estimated to be less than 1/100,000 [4]. Severe GALE deficiency is the least common form of galactosemia with a total of eight patients from two consanguineous families having been detected [4]. The confirmatory diagnosis for these two types of galactosemia often require radioactive assays, which are equally labor intensive and time consuming as the radioactive GALT assay.

Biochemical and clinical heterogeneity among GALT deficiency patients has been well recognized and evidence suggests a strong correlation between clinical presentation and residual GALT activity [6,7,8]. Residual erythrocyte GALT activities in some patients with non-classical or clinical variant galactosemia have been found to be in the range of 1 to 5% of normal control value. Thus, reliable measurement of GALT activity in this range is highly desirable and could be critical in the study and delineation of biochemical phenotypes in galactosemic patients since over 230 GALT gene mutations have been identified [4,9]. To this end, we recently developed an LC-MS/MS based GALT assay [10]. This assay required much less labor than the radioactive assay and yet possessed unprecedented performance in measuring low GALT activity. The liquid chromatography (LC) column used in the assay was Atlantis T3 (100 × 2.1 mm, 3 µm) from Waters (Milford, MA), which was a reversed phase column partially end-capped with polar groups to help bind to polar compounds. An ion pairing solvent system, optimized from what had been previously reported in the literature [11], was employed for the required separation selectivity. Coincidentally, Ko *et al.* reported a similar LC-MS/MS based assay for the measurement of GALT activity along with GALK and GALE at the same time [12]. Interestingly, the LC column used by Ko *et al.* was also manufactured by Waters (Milford, MA) and possesses the same chromatographic feature as the Atlantis T3 column except with smaller particle size (1.8 µm). An ion pairing solvent system was also utilized by Ko *et al.* except that the ion pairing reagent triethylamine was employed in lieu of tributylamine within our study. Noteworthy is that the mass spectrometer was operated in negative-ion electrospray ionization (ESI) mode in both LC-MS/MS assays.

In this study we developed a robust and sensitive LC-MS/MS based GALT and GALK assay. We drastically shortened the LC-MS/MS run time, and using a novel normal phase column, eliminated the need for an ion pairing reagent. We also demonstrated that the assays can be used in the analyses of GALT and GALK activities on dried blood spots (DSB).

Materials and Methods

Chemical and reagents

[$^{13}\text{C}_6$]-Gal, [$^{13}\text{C}_2$]-Gal-1-P (dipotassium salt) and [$^{13}\text{C}_6$]-Gal-1-P (dicyclohexylammonium salt, monohydrate) were purchased from Omicron Biochemicals Inc. (South Bend, IN, USA). Glycine (culture grade) was purchased from Fisher Scientific (Waltham, MA, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA). Millipore filtered Milli-Q water was used in all experiments unless otherwise specified.

Preparation of assay solutions and reagents

Containers used in solvent preparation were not sterilized but they were thoroughly rinsed with Milli-Q water prior to use. Unless otherwise specified, all solutions and reagents were stored at -20°C in order to avoid bacterial contamination and/or decomposition.

Assay buffer—The 0.5 mol/L glycine (pH 7.8) and 0.8 mol/L MOPS (pH 8.7) buffer solutions were prepared by dissolving glycine and MOPS, respectively, in water and adjusting pH to the target pH. The solutions were stored at $2-4^\circ\text{C}$ and aliquoted prior to use by pouring them into a new container (such as an Eppendorf tube or 15 mL centrifuge tube) each time. They are stable for at least one year. Erythrocyte or red blood cell (RBC) lysing buffer was prepared as previously described [10].

Calibration Solutions—Calibrators for GALT and GALK assays were combined and consisted of 6 levels of acetonitrile/water (2/1 (v/v)) solutions containing a constant amount of UDP-*N*-acetylglucosamine at 40 $\mu\text{mol/L}$ (IS for the GALT assay), [$^{13}\text{C}_2$]-Gal-1-P at 50 $\mu\text{mol/L}$ (IS for the GALK assay) and different levels of UDPGal and [$^{13}\text{C}_6$]-Gal-1-P. The calibrator concentrations of UDPGal were 50, 12.5, 3.13, 0.78, 0.195, and 0.045 $\mu\text{mol/L}$; the concentrations of [$^{13}\text{C}_6$]-Gal-1-P were 100, 25, 6.25, 1.56, 0.39, 0.10 $\mu\text{mol/L}$. The calibrators were prepared via serial dilutions (4-fold) of the highest level of calibrator using an acetonitrile/water solution (2/1 (v/v)) containing 40 $\mu\text{mol/L}$ of UDP-*N*-acetylglucosamine and 50 $\mu\text{mol/L}$ of [$^{13}\text{C}_2$]-Gal-1-P as a diluent.

Blood samples and preparation

The procurement and use of blood samples in this study from patients with galactosemia and control subjects were approved by the Institutional Review Board (IRB) of Children's Hospital Boston (CHB). Blood samples from patients with GALT deficiency galactosemia including variants were collected in the out-patient phlebotomy unit of CHB. Samples were washed to obtain an erythrocyte pellet and stored as previously described [10]. The blood sample from the GALK deficient patient was washed in the same manner as the GALT deficiency samples and assayed immediately. The samples from control subjects were anonymous samples collected from presumed non-galactosemic individuals; they were washed and used within 24 hours of collection. One volume of washed erythrocyte pellet was lysed with 3 volumes of lysing buffer (freshly prepared aqueous solution containing 12.8 mmol/L NaH_2PO_4 and 3.7 mmol/L of dithiothreitol at pH 7). The hemolysate was incubated at 37°C for 10 min followed by hemoglobin determination using a hemoglobin measurement kit from Pointe Scientific (Canton, MI, USA). The hemolysate (containing ~ 7 g/dL of hemoglobin) was used directly in a standard assay without further dilution.

Assay procedure

The assay strategies for GALT and GALK enzymes are depicted in Fig. 1. The procedures are as follows.

GALT assay—The final GALT assay conditions were identical to our previous GALT assay except some minor changes in the procedure [6]. First, 1 volume of each of 0.5 mol/L glycine buffer (pH 8.7), 2.0 mmol/L UDPGlc, 8.0 mmol/L [$^{13}\text{C}_6$]-Gal-1-P, and lysing buffer were added to a 1.5 mL microcentrifuge tube. The tube was vortexed thoroughly and centrifuged for 10 seconds at room temperature to bring the solution to the bottom of the tube. It was then dispensed to 1.5 mL microcentrifuge tubes chilled on ice, 32 μL per tube. Then, 8 μL of ice-chilled hemolysate of each sample was added to two tubes, one of which was incubated sequentially in boiling water for 3 min, then 37 °C water for 30 min to serve as a blank control. The other tube was incubated in 37 °C water for 30 min and then in boiling water for 3 min. Upon completion of the incubation, 160 μL of acetonitrile IS solution containing 75 $\mu\text{mol/L}$ of [$^{13}\text{C}_2$]-Gal-1-P and 60 $\mu\text{mol/L}$ of UDP-*N*-acetylglucosamine and 40 μL of water were added. The tubes were thoroughly mixed and centrifuged at 13,200 rpm (Eppendorf, model#5415 D, 16,000g) for 10 min. Then, 140 μL of supernatant was transferred to an autosampler vial (Waters, Milford, MA, USA) for LC-MS/MS analysis.

GALK assay—1 volume of each of 2.0 mmol/L of [$^{13}\text{C}_6$]-galactose, 0.8 mol/L of MOPS buffer (pH 7.8), and assay co-factor cocktail (aqueous solution containing 30, 32, and 32 mmol/L of ATP, MgCl_2 , and NaF, respectively) was added to a 1.5 mL microcentrifuge tube. The tube was vortexed thoroughly, centrifuged for 10 seconds at room temperature, and then dispensed to 1.5 mL microcentrifuge tubes chilled on ice, 37.5 μL per tube. Then, 25 μL of ice-chilled hemolysate of each blood sample was added to two tubes, one of which was sequentially incubated in boiling water for 3 min and then in 37°C water for 2 hours to serve as a blank control. The other tube was incubated in 37°C water for 2 hours and boiling water for 3 min. Upon completion of the incubation, 125 μL of IS solution was added to each tube. The tubes were then processed the same way as those in GALT assay in preparation for LC-MS/MS analysis.

LC-MS/MS analysis of erythrocyte GALT and GALK activity

A Waters Quattro Premier mass spectrometer equipped with a Waters Acquity UPLC system (Waters, Milford, MA, USA) was used in this study. GALT and GALK assay samples were run separately but with identical LC-MS/MS conditions. Both the LC column (Waters, BEH amide column, 2.1 $\times$ 50 mm, 1.7 μm) and the autosampler were run at room temperature. Solvent A was 5 mmol/L ammonium formate in acetonitrile/water (90/10 (v/v)); solvent B was 5 mmol/L ammonium formate in acetonitrile/water (50/50 (v/v)). The LC gradient had a constant flow rate at 0.4 mL/min with linear solvent changes between the two consecutive time points. The gradient started with 85% A, changed to 0% A at 1.50 min, maintained at 0% A until 2.70 min. At 2.71 min it changed back to 85% A and remained 85% A until the end of the gradient (3.0 min). To minimize the solvent gaining entry into the mass spectrometer the LC eluent was diverted to waste during time intervals 0 – 1.56 and 1.96 – 3.0 min respectively.

Two injections, one of which was for the blank control, were performed for each sample. The injection volume was 20 μL per analysis. The mass spectrometer was operated in negative-ion ESI mode with multiple reaction monitoring (MRM) scanning. The MS/MS settings were optimized as in our previous GALT assay. The details are summarized in the Supplemental Data Table 1.

LC-MS/MS peak areas were integrated using Waters Masslynx 4.0 software. The peak area ratio of analyte-to-IS ($[^{13}\text{C}_6]$ -UDPGal to UDP-*N*-acetylglucosamine in GALT assay; $[^{13}\text{C}_6]$ -Gal-1-P to $[^{13}\text{C}_2]$ -Gal-1-P in GALK assay) were calculated using Microsoft Excel. The background (the peak area ratio of analyte-to-IS in the blank control tube) was subtracted from the standard assay tube to obtain net analyte-to-IS ratios. The amount of enzymatic product $[^{13}\text{C}_6]$ -UDPGal and $[^{13}\text{C}_6]$ -Gal-1-P produced by GALT and GALK, respectively, were then determined using the corresponding calibration curve. The calibration curve was generated by plotting the peak area ratio of analyte-to-IS against molar ratio of analyte-to-IS obtained from the six levels of calibrators that were run with each batch of samples. The chromatograms presented in Figure 2 were constructed using Igor Pro 5.0 (Wavemetrics Inc, Lake Oswego, OR, USA).

Optimization of the GALK reaction conditions

The substrate concentration, buffer, incubation time and amount of hemolysate in the GALT assay were the same as in our previously reported GALT assay [10]. GALK assay conditions were optimized using the previously reported high pressure liquid chromatography (HPLC) based GALK assay as a starting point [13]. The assay conditions were examined by running the assay at different substrate concentrations (0.025, 0.05, 0.1, 0.2, 0.4, 0.8, and 1.6 mmol/L of $[^{13}\text{C}_6]$ -galactose). Erythrocytes from 2 different individuals were used in this study with one measurement per substrate concentration per erythrocyte sample. The data were fit into Michaelis–Menten equation to obtain the *K_m* of $[^{13}\text{C}_6]$ -galactose. Furthermore, GALK assay were carried out with different incubation times for 0, 0.25, 0.5, 1.0, 2.0, and 2.5 hours, and in a separate experiment with different amounts of hemoglobin at 0, 0.19, 0.38, 0.75, 1.13, 1.5, and 3 mg with two replicates per data point. The amount of GALK product yielded as function of incubation time and amount of hemoglobin was then determined.

Analytical validation of the LC-MS/MS protocol

Ion suppression and recovery—Ion suppression was assessed by comparing the mass spectrometric (MS) signal (peak areas) of the internal standards in neat calibrator solutions to the response of the same solutions with the standard GALT and GALK assays originated from 6 different individuals. The recoveries of the two assays were evaluated by spiking a known amount of enzymatic product into the respective matrix at two levels, and comparing the measured concentrations with the theoretical values. The two levels were equivalent to the amount of enzymatic product produced by the target enzyme with 100% and 1% activity of normal control. More specifically, 7.0 and 0.07 nmol of UDPGal were respectively spiked into a standard GALT assay sample in which substrate UDPGlc was omitted. For the GALK assay, 3 and 0.03 nmol of $[^{13}\text{C}_6]$ -Gal-1-P were respectively spiked into a standard GALK assay sample in which the reaction was stopped at time zero by boiling for 3 min. The recoveries of both GALT and GALK were evaluated using blood samples from 5 different individuals.

Linearity and imprecision—Linearity of the calibration curve and inter-assay variability were evaluated by examining randomly selected calibration curves performed with each batch of samples in 5 different days. To determine the assay imprecision at different levels, a blood sample from a non-galactosemic individual was washed and the packed erythrocytes were aliquoted and stored at -20°C . Starting from day zero (the day when the blood sample was collected and washed) one aliquot was lysed and measured for GALT and GALK activity at four different levels (100, 25, 5 and 0.5% of normal control values), three replicates per level. The three lower levels (25, 5, and 0.5%) were reconstituted from the 100% level by diluting with a lysing buffer. This study was performed on five consecutive days.

Method comparison—The GALT assay was compared with a fluorescent assay [14] by running both assays in parallel using blood samples from 10 normal controls, 7 classical galactosemia, and 6 variants.

Enzyme stability at different storage conditions

Blood samples from three presumed non-galactosemic individuals were collected in heparin tubes. They were aliquoted and stored in the following conditions: un-washed whole blood stored at room temperature; washed RBC pellet stored at 4°C and –20°C. The GALK and GALT activities were measured on day zero, 2, 9, and 16 with one replicate per blood sample per storage condition.

Dried blood spots assay

Dried blood spots (DBS) on a filter paper card (S & S 903) was prepared by spotting whole blood from a presumed non-galactosemic individual on a NBS card, and air drying it at room temperature for 2 days. The DBS filter paper was cut into small pieces using scissors. GALT assays were performed using the small DBS cuts (weighing from 0.31 to 0.94 mg) in place of hemolysate in the standard GALT assay procedure. Likewise, the GALK assay was performed using the small DBS cuts (weighing from 0.70 to 1.91 mg) to substitute for hemolysate in a standard GALK procedure.

Results

LC-MS/MS chromatogram

Identical LC-MS/MS conditions were used in both GALT and GALK assays. The MS/MS transitions monitored in both assays were 606 > 385 for UDP-*N*-acetylglucosamine, 565 > 323 for UDPGal/UDPGlc, 571 > 323 for [¹³C₆]-UDPGal, 265 > 79 for [¹³C₆]-Gal-1-P, 261 > 79 for [¹³C₂]-Gal-1-P, and 259 > 79 for unlabeled hexose monophosphate, respectively. The chromatograms of typical GALT and GALK assays of a normal control and enzyme deficient sample are shown in Fig. 2. As shown, all analytes and IS were detected between 1.6 and 1.9 min. Both [¹³C₆]-Gal-1-P and [¹³C₂]-Gal-1-P had the same retention time, which provides important information for identifying the right GALK enzymatic product when additional peaks are present (see discussion below). None of these transitions detected any additional major peaks other than the target compound, except for the transition 265 > 79 which typically had an additional peak approximately 0.2 min after [¹³C₆]-Gal-1-P (lower chromatogram of Fig. 2. d). This additional peak was found to be from the hemolysate but did not offer any interference due to its chromatographic separation from the targeted [¹³C₆]-Gal-1-P product. In addition, by referencing the retention time of [¹³C₂]-Gal-1-P this additional peak can be readily distinguished from the true peak for [¹³C₆]-Gal-1-P, even if the latter is produced at relatively smaller concentrations. The UDPGal and UDPGlc co-eluted under the devised chromatographic conditions and possessed indistinguishable MS/MS fragmentation profiles. [¹³C₆]-UDPGal also had the same retention time as UDPGal/UDPGlc, which is useful for the identification of the [¹³C₆]-UDPGal peak when samples with minute GALT activity were assayed.

A small peak of [¹³C₆]-UDPGal (<10% of the standard reaction) was typically observed in the blank control of the GALT assay (data not shown). The presence of [¹³C₆]-UDPGal peak in the blank reaction is believed to be caused by the fact that the reaction was not completely halted after the hemolysate was added to the assay and before it was inactivated by heating in boiling water. Noteworthy is that the [¹³C₆]-UDPGal peak was absent in the blank reaction when GALT activity was zero (or non-detectable, see lower chromatograph of Fig. 1 b). This low background in the mass transition for [¹³C₆]-UDPGal provided us the opportunity of reliably measuring low GALT activity.

Optimization for GALK reaction conditions

Optimal substrate concentration ($[^{13}\text{C}_6]$ -galactose) and linearity with time and protein concentration were determined for the GALK assay. The minimum $[^{13}\text{C}_6]$ -galactose concentration to achieve maximum GALK enzyme activity was determined to be 0.4 mmol/L. The effect of $[^{13}\text{C}_6]$ -Gal-1-P on GALT activity followed a typical Michaelis–Menten kinetics trend, with a K_m of 0.078 mM for $[^{13}\text{C}_6]$ -galactose (Fig. 3). The amount of enzymatic product was proportional to the incubation time (up to 2.5 hour) and the amount of protein (up to 3.0 mg of hemoglobin) (see Supplemental Data Fig. 1 and 2). In subsequent experiments, the assay was carried out with 0.4 mmol/L of $[^{13}\text{C}_6]$ -galactose, hemolysate with approximately 1.8 mg of hemoglobin and an incubation time of 2 hours.

Ion suppression and recovery

The MS signal of 40 $\mu\text{mol/L}$ of UDP-*N*-acetylglucosamine in both the calibrators and GALT assayed patient samples was compared, with no statistical difference observed ($N = 6$, $p = 0.26$). The MS signal of 50 $\mu\text{mol/L}$ of $[^{13}\text{C}_2]$ -Gal-1-P in both the calibrators and GALK assayed patient samples were also compared and no statistical difference was observed either ($N = 6$, $p = 0.17$). In the recovery study, the recovery of UDPGal was $98 \pm 3\%$ and $105 \pm 4\%$ (mean $\pm$ SD) when 7.0 and 0.07 nmol of UDPGal were respectively spiked into 5 different GALT assay matrices. The average recovery of $[^{13}\text{C}_6]$ -Gal-1-P were $99 \pm 2\%$ and $104 \pm 4\%$ when 3 and 0.03 nmol of $[^{13}\text{C}_6]$ -Gal-1-P were respectively spiked into 5 different GALK assay matrices. This indicated that the LC conditions provided adequate cleanup and the signals of IS and analytes were not affected by the matrix in the assay, demonstrating the appropriateness of using a standard curve constructed from the calibrators for the quantification of the enzymatic product.

Linearity and imprecision

Calibration curve was established for each batch of samples by performing analysis of the calibrators outlined in the *Materials and Methods* section. The calibration curve in the GALT assay showed linearity from 0.045 to 50 $\mu\text{mol/L}$ of UDPGal (slope = 1.86 ± 0.1 ; $R^2 = 0.9973 \pm 0.0026$) (mean $\pm$ SD, $N = 5$, inter-day). The linearity range (0.045 to 50 $\mu\text{mol/L}$) corresponded to GALT activity of 0.04 to 43 $\mu\text{mol} \cdot (\text{g Hgb})^{-1} \cdot \text{hr}^{-1}$ (or 0.2% to 180% of GALT activity in normal control). In the GALK assay, the calibration curve was linear from 0.1 to 100 $\mu\text{mol/L}$ of $[^{13}\text{C}_6]$ -Gal-1-P (slope = 1.02 ± 0.05 , $R^2 = 0.9994 \pm 0.0009$), corresponding to GALK activity of 0.05 to 5 $\mu\text{mol} \cdot (\text{g Hgb})^{-1} \cdot \text{hr}^{-1}$ (or 0.3 to 250% of GALK activity in normal control). The standard curve in the GALK assay was observed to be more consistent from day to day than the GALT assay due to the employment of isotope labeled IS that is structurally identical to analyte.

In the imprecision study, at levels of 100, 25, 5 and 0.5% of normal enzyme activity, intra-assay CVs of the GALT assay were calculated to be 6.5, 7.6, 7.9, and 9.5%, respectively ($N = 3$); inter-assay CVs were 7.1, 8.7, 9.2, and 16.2% ($N = 5$), respectively. In the GALK assay, the intra-assay CVs were 1.5, 2.1, 2.7, and 9.5%, respectively ($N = 3$); inter-assay CVs were 3.1, 4.4, 5.3, and 12.8% ($N = 3$), respectively. Only 3 days were performed for the GALK inter-day imprecision study due to the instability of GALK enzyme (see discussion below).

Method comparison—Good correlation was observed between the LC-MS/MS and fluorescent assay values (Fig. 5). The 10 control subjects showed a mean $\pm$ SD GALT activity of 27.3 ± 4.6 and 24.2 ± 3.3 $\mu\text{mol} \cdot (\text{g Hgb})^{-1} \cdot \text{hr}^{-1}$ (mean $\pm$ SD) when determined by the fluorescent and LC-MS/MS assays, respectively, with the former being ~13% higher. A similar trend was observed in the 6 samples of variant galactosemia. However, when the samples from 7 patients with classical galactosemia were analyzed, the LC-MS/MS showed

no detectable activity, whereas the fluorescent assay showed non-zero, either positive or negative, values due to the inadequate sensitivity of the fluorescent assay.

Enzyme stability at different storage conditions

The results of the GALT and GALK stability study are presented in Fig. 4. The GALT enzyme showed excellent stability in all of the three storage conditions: whole blood with sodium heparin stored at room temperature, washed erythrocytes stored at 4 °C and –20 °C (Fig. 4 a). No decline in the GALT activity was observed in washed erythrocytes stored at –20 °C for 16 days. A slight and similar decline was observed when stored as whole blood with sodium heparin at room temperature, or as washed packed erythrocytes stored at 4°C. In both storage conditions, approximately 18% GALT activity was lost after 16 days. The GALK enzyme was less stable than GALT. After being stored for 7 and 16 days, the GALK enzyme lost 92 and 98%, respectively, of its activity when stored as whole blood with sodium heparin at room temperature. It lost 25 and 51% when store as washed packed erythrocytes at 4°C, and 59 and 74% as washed packed erythrocytes at –20°C. It showed that GALK enzyme from a normal control subject was stable for at least 2 days when stored in any of the 3 conditions.

Enzyme activities of controls and enzyme deficient patients

The GALT and GALK enzyme activities of normal controls and enzyme deficient subjects are presented in Table 1. Thirty-three normal control samples were analyzed for both GALT and GALK activities by the LC-MS/MS assays developed in this study. The mean $\pm$ SD GALT activity was $23.4 \pm 3.97 \mu\text{mol} \cdot (\text{g Hgb})^{-1} \cdot \text{hr}^{-1}$, which is consistent with the result obtained with our previous LC-MS/MS based GALT assay ($23.8 \pm 3.8 \mu\text{mol} \cdot (\text{g Hgb})^{-1} \cdot \text{hr}^{-1}$ for 71 normal controls) [6]. The mean $\pm$ SD GALK activity of the 33 normal controls was $1.8 \pm 0.43 \mu\text{mol} \cdot (\text{g Hgb})^{-1} \cdot \text{hr}^{-1}$. A total of 30 GALT deficient samples were analyzed. There were 16 samples from patients with severe galactosemia assessed on the basis of clinical presentation and/or laboratory testing, among which 12 had no detectable GALT activity while 4 had activity ranging from 0.07 – $0.20 \mu\text{mol} \cdot (\text{g Hgb})^{-1} \cdot \text{hr}^{-1}$. These four samples with detectable GALT activities had the following genotypes: Q188R/S315L, Q188R/K334R, R148Q/N314D/K334R, and IVS4+7 A>C/unknown mutation. Of note is that none of the samples with the Q188R/Q188R mutation had any detectable activity. The only GALK deficient sample we procured had no detectable GALK activity (Fig. 2 d).

Dried blood spot based assay

The amount of enzymatic product displayed a linear relationship with the amount of DBS, with R^2 of linear fitting being 0.92 and 0.89 in the GALT and GALK assays, respectively (Fig. 6). The imperfect correlation was presumably caused by the uneven distribution of blood on the filter paper, especially when DBS cuts used in this experiment were so small. The largest amount DBS used in the GALT and GALK assays were 0.94 and 1.91 mg, respectively. For comparison, a 3.2 mm DBS disc, the typical size used in the fluorescent NBS assay for galactosemia [15], weighed ~3 mg.

Discussion

The LC-MS/MS based assays for GALT and GALK are principally the same as radioactive assays in that isotope labeled enzymatic products produced from exogenously added isotope labeled substrates by the target enzymes are determined without reliance upon coupled enzymatic reactions. The enzymatic products in our GALT and GALK assays are [$^{13}\text{C}_6$]-UDPGal and [$^{13}\text{C}_6$]-Gal-1-P, respectively. From a separation scientist prospective, the LC-MS/MS analysis of extremely polar compounds, like hexose-monophosphate and UDPGal/UDPGlc, in complex mixtures presents unique analytical challenges. These metabolites do

not bind to reversed phase LC columns under regular conditions, and yet conversely bind to normal phase columns with such affinity that MS incompatible salt containing solvent would be needed for elution. To cope with this issue, ion pairing solvent systems were developed, and have been used in a number of studies before our previously reported LC-MS/MS based GALT enzyme assay [11,16,17]. The ion pairing reagents used in these studies are amine derivatives, such as tributylamine, triethylamine, and octylamine. These amine derivatives can be readily protonated to become positively charged, resulting in very high sensitivity in positive-ion ESI mode but almost non-detectable in negative-ion ESI mode in MS analyses. The LC-MS/MS based GALT assays that were independently developed in tandem by our group and Ko *et al.* used a very similar LC column and ion pairing solvent system, which to some extent illustrates the limited options for the LC-MS/MS analysis of the hexose monophosphates and UDPGal/UDPGlc [12].

With the employment of the amide column that was recently launched by Waters, we were able to achieve adequate sample cleanup and specificity using the common LC-MS solvent system that consisted of acetonitrile, water, and ammonium formate at 5 mmol/L level. With the new solvent system, the sensitivity of Gal-1-P and UDPGal/UDPGlc on MS was decreased by approximately an order of magnitude compared to the ion pairing solvent system in our previously reported assay. The sensitivity loss was compensated by minimizing dilution prior to MS analysis. In our previously reported GALT assay, the GALT sample was diluted by 40 times overall prior to MS analysis, whereas in this improved assay it was only diluted by 6 times. With this adjustment, we achieved the same limit of quantitation (LOQ) of $0.05 \mu\text{mol}\cdot(\text{g Hgb})^{-1}\cdot\text{hr}^{-1}$ or 0.2% of normal control values as we had in the previous GALT assay [10]. The imprecision of the improved GALT assay was comparable to that of our previously reported GALT assay. The intra- and inter-assay CVs were all within 10% when GALT activity was down to 5% of normal control values. Even at 0.2% of normal control value, they were < 20%. This extremely low LOQ proved to be useful for measuring enzyme activity that is less than 1% of normal control values. In this study, 4 out of 16 patients with severe galactosemia were determined to have erythrocyte GALT activity ranging from 0.07 to $0.2 \mu\text{mol}\cdot(\text{g Hgb})^{-1}\cdot\text{hr}^{-1}$ (or 0.3 to 0.8% of normal control values). Among these 4 patients one with Q188R/S135L mutation had $0.11 \mu\text{mol}\cdot(\text{g Hgb})^{-1}\cdot\text{hr}^{-1}$ GALT activity. A similar result was reported for a patient who was homozygous for S135L, a prevalent mutation in African-Americans and native South Africans [18]. Somewhat unexpectedly, we measured erythrocyte GALT activity in 25 classic galactosemic patients with the Q188R/Q188R mutation during this and previous studies and none of them had any detectable activity. This discordance with literature in which minute residual GALT activity was sometimes reported is believed to be caused by insufficient sensitivity of the methods for measuring low GALT activity in the literature [18].

The GALK assay described here has a similar assay procedure and the same LC-MS/MS settings as the GALT assay. The major difference is that the former assay requires a larger amount of protein and a longer incubation time due to the much lower GALK activity than GALT activity. The mean $\pm$ SD GALK activity in erythrocytes of 33 controls (>1 year old children) was determined to be $1.8 \pm 0.43 \mu\text{mol}\cdot(\text{g Hgb})^{-1}\cdot\text{hr}^{-1}$, which is in the same range as the radioactive assay reported by Emory Genetics Laboratory (<http://genetics.emory.edu/egl/tests/>). This value is also consistent with what was reported by Ko *et al.* who had a mean of $1.7 \mu\text{mol}\cdot(\text{g Hgb})^{-1}\cdot\text{hr}^{-1}$ for ~ 30 controls determined by their LC-MS/MS assay[12].

Besides being used as diagnostic assays in biochemical genetics laboratories, this LC-MS/MS based GALT and GALK assays may be considered for second tier testing in the newborn screening laboratories. In the current NBS for galactosemia, a two tiered screening modality is performed in most of the states in the US. Total galactose in a DBS specimen is

first measured by a fluorescent assay. If galactose is above the cutoff value, GALT activity will be measured to determine if a newborn is GALT deficient. In rare situations, if total galactose is elevated and GALT activity is normal, GALK and/or GALE activity may need to be measured. In some states, the fluorescent GALT assay is concurrently performed with the total galactose measurement as a first-tier screening test.

The feasibility of measuring DBS GALT activity by the LC-MS/MS assay was demonstrated in this study. We demonstrated that the amount of enzymatic product displayed a linear relationship with the size of the DBS. Theoretically, this assay may better predict the phenotype of a subject with clinical variant GALT deficiency due to the sensitivity and specificity of this LC-MS/MS based method. Understandably, this might eliminate the need for a second and third DBS and hasten the referral of the infant to a biochemical genetics unit. Furthermore, the GALK assay could be employed to examine the condition of a high galactose level and normal GALT activity. These benefits would need to be carefully studied in a large scale pilot study, which is beyond the scope of our work which is focused on the generation of optimal assays for the biochemical genetics laboratories.

In conclusion, we have developed an improved LC-MS/MS based GALT assay in which we eliminated the use of ion pairing reagents as in the previously reported LC-MS/MS GALT assays [10,12] to make the assay more robust and suitable for routine use. Meanwhile, we have radically cut down the instrument run time to 3 min to save valuable instrument time. Furthermore, a robust and sensitive LC-MS/MS based GALK assay was also developed, similarly, without the use of an ion pairing reagent. The feasibility of using the LC-MS/MS based assays for the analysis of GALT and GALK activities on DBS were demonstrated, which opens the possibility of adapting them as second tier tests for NBS of galactosemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was funded in part by a research grant from the Parents of Galactosemic Children, USA, and in part by the grant UL1 RR025758-01 from the National Center for Research Resources, National Institutes of Health, to the Harvard Catalyst Clinical & Translational Science Center (Harvard Catalyst). The authors thank the CTSU staff of Children's Hospital Boston for their superb assistance and the patients with galactosemia who traveled to Boston from around the U.S.A. and participated in this study.

Abbreviations

Gal-1-P	galactose-1-phosphate
GALE	uridine diphosphate galactose 4'-epimerase
GALK	galactokinase
GALT	galactose-1-phosphate uridylyltransferase
Glu-1-P	glucose-1-phosphate
LC-MS/MS	liquid chromatography tandem mass spectrometry
MOPS	3-(N-morpholino)-propanesulfonic acid
NBS	newborn screening
UDPGal	uridine diphosphate galactose

[¹³C₆]-UDPGal	¹³ C ₆ labeled uridine diphosphate galactose
UDPGlc	uridine diphosphate glucose

References

1. National Newborn Screening and Genetics Resource Center. National Newborn Screening Report, 1999. Austin, TX: National Newborn Screening and Genetics Resource Center; 2002.
2. Segal, S.; Berry, GT. Disorders of galactose metabolism. In: Scriver, CR.; Beaud, A.; Sly, WS.; Valle, D., editors. The metabolic and molecular bases of inherited disease. 8th ed.. New York: McGraw-Hill; 2001. p. 967-1000.
3. Berry, S.; Segal, GT.; Gitzelmann, R. Disorders of galactose metabolism. In: Fernandes, J.; Saudubray, JM.; Berghe, G.; Walter, JW., editors. Inborn Metabolic Diseases. 4th ed.. Springer; 2006. p. 121-130.
4. Fridovich-Keil, JL.; Walter, JH. Galactosemia. Chapter 72. In: Valle, D.; Beaudet, A.; Vogelstein, B.; Kinzler, BW.; Antonarakis, SE.; Ballabio, A.; Scriver, CR., editors. The online metabolic and molecular bases of inherited disease. 9th ed.. New York: McGraw-Hill; 2008.
5. Shin-Buehring YS, Schaub J. Pitfalls in the radioactive method of galactose-1-phosphate uridylyltransferase activity measurement. Clin. Chim. Acta. 1980; 106:231–234. [PubMed: 6250747]
6. Hsia DY, Walker FA. Variability in the clinical manifestations of galactosemia. J. Pediatr. 1961; 59:872–883. [PubMed: 14449515]
7. Ng WG, Bergren WR, Donnell GN. Galactose-1-phosphate uridylyltransferase activity in galactosemia. Nature. 1964; 203:845–847. [PubMed: 14204065]
8. Waggoner DD, Buist NR, Donnell GN. Long term prognosis in galactosemia: results of a survey of 350 cases. J. Inherit. Metab. Dis. 1990; 13:802–818. [PubMed: 1706789]
9. Caderon FR, Phansalkar AR, Crockett DK, Miller M, Mao R. Mutation database for the galactose-1-phosphate uridylyltransferase (GALT) gene. Human Mutat. 2007; 28:939–934. ARUP Laboratories. Galactose-1-phosphate Uridylyltransferase (GALT) http://arup.utah.edu/database/galactosemia/GALT_welcome.php.
10. Li Y, Ptolemy AS, Harmonay L, Kellogg M, Berry GT. Quantification of galactose-1-phosphate uridylyltransferase enzyme activity by liquid chromatography-tandem mass spectrometry. Clin. Chem. 2010; 56:772–780. [PubMed: 20348403]
11. Luo B, Groenke K, Takors R, Wandrey C, Oldiges M. Simultaneous determination of multiple intracellular metabolites in glycolysis, pentose phosphate pathway and tricarboxylic acid cycle by liquid chromatography-mass spectrometry. J. Chromatogr. A. 2007; 1147:153–164. [PubMed: 17376459]
12. Ko D, Jun S, Park H, Son S, Jin KU, Kim JQ, Song Y, Song J. Multiplex enzyme assay for galactosemia using ultraperformance liquid chromatography–tandem mass spectrometry. Clin. Chem. 2010; 56:764–771. [PubMed: 20299679]
13. Mizoguchi N, Eguchi T, Sakura N, Ueda K. Erythrocyte galactokinase assay with high performance liquid chromatography. Clin. Chim. Acta. 1993; 216:145–151. [PubMed: 8222265]
14. Beutler E, Baluda MC. Improved method for measuring galactose-1-phosphate uridylyltransferase activity of erythrocytes. Clin. Chim. Acta. 1966; 13:369–379. [PubMed: 5943827]
15. Fujimoto A, Okano Y, Miyagi T, Isshiki G, Oura T. Quantitative Beutler test for newborn mass screening of galactosemia using a fluorometric microplate reader. Clin. Chem. 2000; 46:806–810. [PubMed: 10839768]
16. Karlsson AÅ, Michelsen P, Larsen Å, Odham G. Normal-phase liquid chromatography class separation and species determination of phospholipids utilizing electrospray mass spectrometry/tandem mass spectrometry. Rapid Comm. Mass Spec. 1996; 10:775–780.
17. Wamelink MM, Struys EA, Huck JH, Roos B, Van der Knaap MS, Jakobs C, Verhoeven NM. Quantification of sugar phosphate intermediates of the pentose phosphate pathway by LC–MS/MS: application to two new inherited defects of metabolism. J. Chromatogr. B. 2005; 823:18–25.

18. Lai K, Langley SD, Singh RH, Dembure PP, Hjelm LN, Elsas LJ LJ. A prevalent mutation for galactosemia among black Americans. *J. Pediatr.* 1996; 128:89–95. [PubMed: 8551426]

Fig. 1. Assay strategies for GALT and GALK assays

Enzymatic products, [$^{13}\text{C}_6$]-UDPGal in GALT assay and [$^{13}\text{C}_6$]-Gal-1-P in GALK assay, were determined by LC-MS/MS, using UDP-*N*-acetylglucosamine and [$^{13}\text{C}_2$]-Gal-1-P, respectively, as an internal standard.

Fig. 2. LC-MS/MS chromatograms of GALT and GALK assays

GALT assay using hemolysate from a normal control (a) and classic galactosemic patient (b), and GALK assay using hemolysate from a normal control (c) and GALK deficiency subject (d). The IS in GALT assay (UDP-*N*-acetylglucosamine) was detected by 606 > 385, and its analyte ($[^{13}\text{C}_6]\text{-UDPGal}$) was detected by 571 > 323. The IS in GALK assay ($[^{13}\text{C}_2]\text{-Gal-1-P}$) was detected by 261 > 79, and its analyte ($[^{13}\text{C}_6]\text{-Gal-1-P}$) was detected by 265 > 79. On the bottom chromatogram of panel (d), the analyte peak of GALK assay (retention time at 1.72 min) was absent; the partially displayed peak (*) was an impurity from hemolysate. Other MS/MS transitions that are not used in the calculation of enzyme activity were monitored mainly for informational purpose; their chromatograms are not shown.

Fig. 3. GALK activity in relation to $[^{13}\text{C}_6]$ -galactose concentration

Each data point represents a single measurement at the corresponding substrate concentration.

Fig. 4. Stability of erythrocyte GALT and GALK enzymes in different storage conditions
 Each data point represents the average of 3 measurements of 3 individual samples, one measurement per sample. The three storage conditions: whole blood with sodium heparin stored at room temperature, washed packed erythrocytes stored at 4 °C and -20°C are represented by symbol of square, star, and diamond, respectively, in (a) for GALT and (b) for GALK enzyme activities. The measured enzyme activities were normalized to that of day zero of the corresponding sample. The activities of day zero were set as 1.

Fig. 5. GALT activities determined by the LC-MS/MS assay and a reference fluorescent assay
 GALT activity was expressed in unit of $\mu\text{mol} \cdot (\text{g Hgb})^{-1} \cdot \text{hr}^{-1}$

Fig. 6. The amounts of GALT (a) and GALK (b) enzymatic products in response to the weight (size) of DBS

Table. 1

The unit of enzyme activity was $\mu\text{mol}\cdot(\text{g Hgb})^{-1}\cdot\text{hr}^{-1}$

GALT and GALK Activity in Normal Control and Enzyme Deficient Subjects				
GALT			GALK	
Control (n=33)	Severe Galactosemia ** (n = 16)	Variant/Carrier ** (n = 14)	Control (n = 33)	GALK deficient (n = 1)
23.4+/-3.97 (mean $\pm$ SD)	ND * (n = 12)	0.51-15.5 (n = 14)	1.8 $\pm$ 0.43 (mean $\pm$ SD)	ND
15.4 - 32.2 (min - max)	0.07-0.20 (n = 4)		1.0-2.7 (min-max)	

* ND: Non-detectable with LOD of 0.1% of normal controls (or $0.02 \mu\text{mol}\cdot(\text{g Hgb})^{-1}\cdot\text{hr}^{-1}$).

N/A: not applicable.

** Gene mutation of each sample can be found in Supplemental Data Table 2.