Multiplex Enzyme Assay for Galactosemia Using Ultraperformance Liquid Chromatography–Tandem Mass Spectrometry

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BACKGROUND: Galactosemia is one of the most important inherited disorders detected by newborn screening tests. Abnormal results in screening tests should be confirmed by enzyme activity assays, but existing methods are time and labor intensive. We developed a novel multiplex enzyme assay for galactosemia using ultraperformance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS).

METHODS: [13C6]-galactose, [13C2]-galactose-1-phosphate, and UDP-glucose were used as substrates for 3 galactose-metabolizing enzymes. The end products from the combined reaction mixtures, [13C6]-galactose-1-phosphate, UDP-[13C2]-galactose, and UDP-galactose, were simultaneously measured using UPLC-MS/MS. Linearity, imprecision, ion suppression, and the effects of substrate were evaluated to determine assay performance. Enzyme activities from 35 healthy individuals, 8 patients with enzyme deficiency, and 18 mutant cells were analyzed.

RESULTS: Substrates, products, and internal standards from the mixture of 3 enzyme reactions were clearly separated by using UPLC-MS/MS, with an injection cycle time of 10 min. Ion suppression was 0.1%–2.5%, the interassay imprecision of UPLC-MS/MS was 3.3%–10.6% CV, and the linearity of each system was good ($R^2 = 0.994–0.999$). Patient samples and mutated cells showed consistently low enzyme activities compared with those of normal individuals and wild-type cells.

CONCLUSIONS: This method allows for a high-throughput and reproducible multiplex enzyme assay for galactosemia in erythrocytes.

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Galactosemia is an autosomal recessive disorder caused by an accumulation of unmetabolized galactose and its derivatives (galactose-1-phosphate and galactitol). Three enzymes responsible for galactosemia have been identified: galactokinase (GALK; EC 2.7.1.6), galactose-1-phosphate uridylyltransferase (GALT; EC 2.7.7.12), and uridine diphosphate (UDP)-galactose-4-epimerase (GALE; EC 5.1.3.2) (1–3). The phenotypic characteristics of galactosemia vary widely according to the defective enzyme and causative genetic alterations (4–6). Many countries perform newborn screening tests for galactosemia because a simple diet restriction can relieve the symptoms of the disease and ameliorate the prognosis. For babies with positive screening results, i.e., increased galactose and/or galactose-1-phosphate, confirmatory analyses should follow. These should include quantitative enzyme assays from fresh whole blood, galactose-1-phosphate analysis in erythrocytes, and mutational analysis of galactokinase 1 (GALK)6, UDP-galactose-4-epimerase (GALE), and galactose-1-phosphate uridylyltransferase (GALT) genes (7). Numerous disease-causing mutations have been identified, more than 200 mutations in GALT, 31 mutations in GALK1, and 20 mutations in GALE (8). Quantitative enzyme analysis is critical, however, because many undiscovered genetic alterations could exist.

Radiometric, spectrophotometric, fluorometric, and HPLC techniques have been applied for the enzyme assays (9–12). In the GALK assay, radiolabeled [14C]-galactose catalysis to [14C]-galactose-1-phosphate by endogenous galactokinase is monitored and quantified by using densitometry after chromatographic separation (9). In the GALT assay, radiolabeled [14C]-

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Nonstandard abbreviations: GALK, galactokinase; GALT, galactose-1-phosphate uridylyltransferase; GALE, UDP-galactose-4-epimerase; UDP, uridine diphosphate; UPLC-MS/MS, ultraperformance liquid chromatography–tandem mass spectrometry; HSS, High Strength Silica; MRM, multiple reaction monitoring.

6 Human genes: GALK1, galactokinase 1; GALE, UDP-galactose-4-epimerase; GALT, galactose-1-phosphate uridylyltransferase.
galactose-1-phosphate is used as a substrate, and the product [14C]-UDP-galactose is isolated by diethylaminoethyl cellulose–thin-layer chromatography and quantified by densitometry (10). In the GALE assay, enzymatic conversion from UDP-galactose to UDP-glucose is detected either by radioactive (9) or nonradioactive assay, with substrates and products separated and quantified by HPLC (11). However, these enzyme assays require the use of radioactive materials, which can be hazardous, and also require multiple reaction steps and long separation times. Spectrophotometric and fluorometric assays have been used for GALT and GALE, but suffer from lower sensitivity (12). All of these methods measure the 3 enzymes separately, requiring much time and labor.

As an alternative, we have developed a novel multiplex enzyme assay for galactosemia using ultraperformance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS).

**Materials and Methods**

HPLC-grade methanol (Burdick and Jackson, Muskegon) and water (Mallinckrodt Baker) were used. Stable isotopes [13C6]-galactose, [13C1]-galactose-1-phosphate, and [13C2]-galactose-1-phosphate were purchased from Omicron Biochemicals. All other reagents were of research grade or better and were purchased from Sigma Chemical. The institutional review board of the Seoul National University Bundang Hospital approved the study.

**STANDARD SOLUTIONS**

The stock solutions containing 10 mmol/L [13C6]-galactose-1-phosphate and 10 mmol/L UDP-galactose were prepared in HPLC-grade water and stored at −70 °C. Standard stock solutions were serially diluted in water to produce working standards. Calibration curves were constructed with 5 concentrations of [13C6]-galactose-1-phosphate (0, 0.1, 0.5, 1.0, and 2.0 mmol/L) and 7 concentrations of UDP-galactose (0, 0.05, 0.1, 0.25, 0.5, 1.0, and 2.0 mmol/L).

**ENZYME ASSAY PROCEDURES**

Whole blood collected in heparin tubes was used for erythrocyte enzyme measurements. After washing 2 times with normal saline, erythrocytes were diluted 3-fold for the GALK assay and 10-fold for the GALE and GALT assays. For the GALE assay, packed erythrocytes were purified with Micro Bio-Spin® P-30 tris chromatography columns (Bio-Rad) to remove all small molecules.

Enzyme reactions are depicted in Fig. 1. Reaction mixtures contained 0.2% saponin, 4 mmol/L NaF, 8 mmol/L MgCl2, 0.2 mol/L Tris-Cl buffer (pH 8.1), 0.8 mmol/L [13C6]-galactose, and 7.5 mmol/L ATP for GALK assay; 2.5 mmol/L [13C2]-galactose-1-phosphate, 2.5 mmol/L UDP-glucose, 0.5 mol/L glycine NaOH (pH 8.7), and 0.04 mol/L cysteine-HCl (pH 8.7) for GALT assay; 5 mmol/L UDP-glucose, 5 mmol/L NAD, and 0.23 mol/L of glycyl-glycine buffer (pH 9.0) for GALE assay. We added 70 μL of each reaction mixture to 30 μL of diluted erythrocytes or working standards, then incubated the mixtures at 37 °C for 30 min (for GALT and GALE assay) or 2 h (for GALK assay). Heat-denatured (95 °C, 5 min) enzyme was used as a blank.

Enzyme reactions were stopped after the predetermined incubation time by heating at 95 °C for 5 min and then centrifuged at 15000g for 10 min. We then combined 10 μL of supernatant from all 3 reaction tubes in a clean tube. To this tube we added 70 μL of mobile phase with 0.06 mmol/L UDP-N-acetylglucosamine and 0.08 mmol/L [13C1]-galactose-1-phosphate as internal standards. The substrates and products in the mixed enzyme reactions were separated and quantified using UPLC-MS/MS. If not otherwise specified, the reaction conditions were identical throughout the study.

The hemoglobin concentration of diluted hemolysates was determined by using a cyan-hemoglobin reagent and an automatic complete blood cell count analyzer (Sysmex).

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**Fig. 1. Metabolic pathway and analytic strategy for 3 galactose-metabolizing enzymes.**

End products of each enzyme reaction, UDP-[13C2]-galactose, [13C6]-galactose-1-phosphate, and UDP-galactose, were measured by UPLC-MS/MS.
UPLC-MS/MS CONDITIONS

UPLC separation was achieved with a Waters ACQUITY UPLC® High Strength Silica (HSS)-T3 Column, 2.1 × 100 mm, 1.8 µm. The mobile phase was a gradient of a mixture of 20 mmol/L triethylamine buffer, pH 5.6 (buffer A), and distilled water. The column was eluted at 250 µL/min by a 0%–100% (vol/vol) gradient of buffer A in water under the following conditions: 0–7.2 min, 100% buffer A; 7.2–9.5 min, 100% water; 9.5–10 min, 100% buffer A. We injected 10 µL of each sample into the HSS T3 column, and performed chromatographic separation at room temperature.

A Quattro Premier XE tandem mass spectrometer (Waters) was operated with the following settings: capillary voltage, 3.6 kV; cone voltage, 35–45 V; collision energy, 22–26 eV depending on analytes (Table 1); collision gas, argon at 3.2 ± 10−3 mbar. Quantification was achieved by multiple reaction monitoring (MRM) in negative-ion mode. We performed integration of peak area and data analysis using QuanLynx 4.0 software.

ION SUPPRESSION

Assessment of matrix effect was performed as previously described with minor modification (5). We added 70 µL of enzyme reaction mixture without substrate to 30 µL of 5 different washed erythrocyte samples. Samples were incubated at 95 °C for 5 min, followed by centrifugation and collection of the supernatant. The supernatants (30 µL) were mixed with 70 µL of the mobile phase spiked with high or low concentrations of UDP-galactose (0.2 mmol/L or 0.02 mmol/L) and [13C6]-galactose-1-phosphate (0.25 mmol/L or 0.025 mmol/L). To estimate the matrix effect, 70 µL of the mobile phase with the same concentrations of UDP-galactose and [13C6]-galactose-1-phosphate was admixed with 30 µL of distilled water. The experiment was repeated 3 times. The mean absolute matrix effect (%) was examined by comparing the MS/MS response (peak areas) obtained from the erythrocyte sample to the MS/MS response of the same analyte present in the mobile phase. The presence of a relative matrix effect was also assessed by direct comparison of the MS/MS responses (peak areas) of an analyte spiked into erythrocyte samples originated from 5 different sources. The variability in these responses, expressed as CVs (%), was considered as a measure of the relative matrix effect.

LINEARITY AND IMPRECISION

Linearity of the calibration curve and interassay variability were evaluated over a concentration range of 0–2 mmol/L for [13C6]-galactose-1-phosphate and UDP-galactose, respectively.

Intrarun imprecision was determined by 10 replicated analyses of randomly selected whole blood samples. To evaluate interassay precision, the specimen was divided into aliquots and stored at −70 °C for the GALT and GALE assays and 4 °C for the GALK assay (membrane-bound GALK enzyme is more stable at 4 °C) (9) and measured for 5 consecutive days.

EFFECT OF SUBSTRATE CONCENTRATION

We calculated $K_m$ values for GALK, GALT, and GALE at various substrate concentrations: 0.08 mmol/L to 1.6 mmol/L [13C6]-galactose for GALK; 0.67 mmol/L to 10 mmol/L UDP-glucose for GALE and 0.2 mmol/L to 5 mmol/L [13C2]-galactose-1-phosphate and 0.2 mmol/L to 5 mmol/L UDP-glucose for GALT. A minimum of 5 points was used for each analysis, and each point was measured 3 times. We fit data to the Michaelis–Menten equation by using a least squares regression and calculated respective $K_m$ values.

EFFECT OF SAMPLE AMOUNTS AND INCUBATION TIME

To evaluate the linearity of the reaction as a function of the amount of enzymes in the assay, we measured the GALK, GALT, and GALE enzyme activities in the erythrocyte samples at 2- to 20-fold, 5- to 40-fold, and 5- to 40-fold dilutions, respectively. Each diluent was analyzed in triplicate. To assess the effect of incubation time, reactions were monitored for 240 min.

ANALYSIS OF PATIENTS AND MUTATED CELLS

To validate the capability of our system, we analyzed 8 patients with confirmed galactosemia (4 with GALE...
deficiency, 3 with GALT deficiency, and 1 with GALK deficiency). All affected patients had positive results in the neonatal total galactose screening test performed by colorimetric enzyme assay on dried-blood spots and underwent radiometric assay testing for enzyme activities in erythrocytes (13, 14). Newborn screening assay on dried blood spots revealed a total mean (SD) galactose concentration of 167 (44) mg/L, and the enzyme activities in erythrocytes were 0.6 μmol/h/g Hb for GALK, 7.5 (4.6) μmol/h/g Hb for GALT, and 4.5 (3.0) μmol/h/g Hb for GALE. We tested 26 healthy individuals tested for their GALT and GALE activities and 35 for GALK activity.


**Comparison with a traditional radiometric assay**

To compare our revised method with a traditional radiometric assay (9), 19 whole bloods with various enzyme activities were selected and tested for their GALT and GALE activities and 33 samples for GALK activities. Passing-Bablok regression analysis was performed.

**Results**

**Materials and Method Development of UPLC-MS/MS**

Galactose-1-phosphate and UDP-galactose produced more than 10 times and 50 times higher signals in negative mode than those in positive mode, respectively. To determine whether the matrix of packed erythrocytes and enzyme reaction mixture caused ion suppression, the standard solution was analyzed with and without the matrix. In terms of absolute matrix effect, negligible amounts of ion suppression were observed for UDP-galactose (2.5% for 0.02 mmol/L and 1.4% for 0.2 mmol/L concentration) and galactose-1-phosphate (2.5% for 0.025 mmol/L and 0.1% for 0.25 mmol/L). The variability values (CVs) calculated from the responses of the 5 different sources were also low (2.4% and 2.0% for 0.02 mmol/L and 0.2 mmol/L UDP-galactose, respectively; 3.8% and 1.7% for 0.025 mmol/L and 0.25 mmol/L galactose-1-phosphate, respectively), suggesting that the relative matrix effect was absent for galactose-1-phosphate and UDP-galactose.

Substrates, products, and internal standards were fully separated by using UPLC with an HSS T3 1.8-μm column (2.1 × 100 mm). We were able to reduce the chromatographic separation time to 7 min, allowing an injection cycle time of 10 min (Fig. 2).

To evaluate the effect of the cross-detection in the channel of the products from endogenous nonlabeled metabolites of the enzyme reaction mixture, we compared the product signal intensity in the presence and absence of substrate. In terms of GALK and GALT enzyme assay, no signal in the channels of products ([13C6]-galactose-1-phosphate and UDP-[13C2]-galactose, respectively) was detected in the reaction mixture in the absence of labeled substrates, suggesting there is no cross-detection in the channel of the products from nonlabeled metabolites. In the GALE enzyme assay, a small background signal was detected in the channel of the product (UDP-galactose) in the reaction mixture without GALE substrate; the intensity of this background signal was only 3.8% that of the background signal detected in the channel of the enzyme reaction in the presence of substrate. Furthermore, the effect of the background intensity could be eliminated because the enzyme activities were calculated by subtracting the amounts of products in a blank reaction containing heat-denatured enzymes from that of the test.

**Linearity and Imprecision**

Interassay calibration variability data obtained for concentrations of 0–2 mmol/L for [13C6]-galactose-1-phosphate and 0–2 mmol/L for UDP-galactose on 5 consecutive days showed a linear and reproducible curve in the observed analytical ranges [intercept of −0.004 (95% CI −0.007 to −0.002), slope of 0.272 (95% CI 0.231–0.313), and correlation coefficient, r², of 0.999 (95% CI 0.998–1.000) for the [13C6]-galactose-1-phosphate; intercept of 0.012 (95% CI −0.015–0.038), slope of 2.049 (95% CI 1.861–2.236), and correlation coefficient, r², of 0.994 (95% CI 0.984–1.004) for the UDP-galactose].

The intraassay imprecision were 3.3%, 9.9%, and 10.6% for GALK, GALT, and GALE assays, respectively. Interassay imprecision were 6.6%, 16.8%, and 19.9% for GALK, GALT, and GALE, respectively.
EFFECT OF SUBSTRATE CONCENTRATION

Fig. 3 depicts the response intensity at different substrate concentrations. For all 3 enzymes, the amount of product showed a hyperbolic dependence on the substrate concentration. $K_m$ values were 0.25 mmol/L for $[^{13}C_6]$-galactose in the GALK assay, 1.81 mmol/L for $[^{13}C_2]$-galactose-1-phosphate and 0.44 mmol/L for UDP-glucose in the GALT assay, and 1.05 mmol/L for UDP-glucose in the GALE assay. Based on these $K_m$ values, we selected a $[^{13}C_6]$-galactose concentration of 0.64 mmol/L for the subsequent GALK assay; $[^{13}C_2]$-galactose-1-phosphate and UDP-glucose concentration of 5 mmol/L and 0.25 mmol/L, respectively, for the GALT assay; and a UDP-glucose concentration of 5 mmol/L for the GALE assay.

EFFECT OF SAMPLE AMOUNTS AND INCUBATION TIME

The amount of product was proportional to the amount of erythrocytes. However, a flattening of the ratio was observed at higher erythrocyte concentrations, especially for GALE (Fig. 4A). This finding was presumably attributable to the presence of one or more inhibitors in the blood, because an increased erythrocyte concentration would lead to an increase in the inhibitor/substrate ratio. We chose a 3-fold dilution of erythrocytes for all subsequent GALK assays, and a 20-fold dilution for GALT and GALE assays.

The product from each enzyme assay increased in a linear fashion during incubation periods of 0–240 min (Fig. 4B). GALK and GALT enzyme assays showed a linear response up to 240 min; GALE showed a linear response up to 120 min. We chose 30 min of incubation time for the GALT and GALE assays, but 120 min for the GALK reaction because of the weak response intensity.

ENZYME ACTIVITY IN PATIENTS AND MUTATED CELLS

Fig. 5 displays the enzyme activities of affected patients and healthy individuals. Patients showed generally low erythrocyte enzyme activity compared with the general
population. In the expression study, mutated cells produced consistently lower enzyme activities than wild-type cells (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol56/issue5).

Method Comparison

Passing and Bablok regression analysis revealed that the UPLC-MS/MS method and the radiometric assay compared favorably for GALK ($r = 0.9421$) and GALT activities ($r = 0.8042$), but generated appreciable differences for GALE activity ($r = 0.6788$) (see online Supplemental Fig. 2).

Discussion

We developed and evaluated a novel and rapid multiplex enzyme assay for galactosemia. Enzyme reactions were simplified to 1 step, and the products from 3 enzyme reactions were measured simultaneously without separation from substrates. The performance of the quantitative test compared to a traditional radiometric method was generally acceptable.

The previously used enzyme assay (9) incorporated a radiometric test, which involves a risk of radiation exposure. Furthermore, manual column separation of products from substrates and reaction mixtures was essential before measurement, and the GALE assay required a 2-step enzyme reaction. These multistep procedures are labor-intensive and time-consuming and carry a greater risk of error owing to their complexity.

To develop a multiplex enzyme assay in a combined reaction mixture, a rapid and simultaneous separation of substrates and products is mandatory. To achieve successful separation, stable isotope compounds ([13C6]-galactose for GALK and [13C2]-galactose-1-phosphate for GALT) were used as substrates (Fig. 1). We could then measure the products ([13C6]-galactose-1-phosphate and [13C2]-UDP-galactose) without any interference from the substrates and products of the other enzyme reactions.

GALE is known to catalyze a freely reversible reaction between UDP-galactose and UDP-glucose, and most enzyme assays use UDP-galactose as a substrate (11, 15, 16). However, because UDP-glucose is used as a GALT substrate in the combined reaction mixture, we could not measure UDP-glucose as a product of GALE activity. Therefore, we adopted a reverse enzyme reaction for the GALE assay, in which UDP-galactose could be measured as a product without any interference. We confirmed that the reverse reaction correlated with the forward reaction, had considerable in-

![Fig. 3. Michaelis–Menten plots of the velocity versus various substrates for GALK, GALT, and GALK enzymes. The experiment was repeated 3 times.](image-url)
tensity, and worked well for the diagnosis of patients and for the enzyme assay in mutant cells. As shown in Fig. 2, UPLC-MS/MS separated the substrates and products in the combined reaction mixture without any interference from the substrates and products of the other enzyme reactions.

We measured UDP-galactose and UDP-[13C2]-galactose as products of the GALE and GALT reactions, respectively. To separate UDP-galactose from UDP-glucose, we adopted ion-pairing methods using 20 mmol/L triethylamine buffer (pH 5.6). Most ion-pairing methods to separate nucleotide sugars use a high concentration of triethylamine, a high flow rate, or a longer separation time (17–19). In this revised system, we could separate UDP-galactose and UDP-glucose in 7 min by using a UPLC HSS T3 1.8-μm column (2.1 × 100 mm) and a relatively low flow rate (0.25 mL/min).

The correlation between our UPLC-MS/MS assay and a traditional radiometric assay was relatively good.

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**Fig. 4. Effect of sample amounts and incubation time.**

(A), Representative plots of the linearity of erythrocyte GALK, GALT and GALE enzyme reaction with respect to the amount of added erythrocytes (indicates inverse value of dilution folds). The experiment was repeated 3 times. (B), Representative plots of the time course of each GALT, GALK, and GALE enzyme reaction. Each enzyme reaction was monitored for 240 min, and the experiment was repeated 3 times.

**Fig. 5. Enzyme activities in affected patients and normal individuals measured using erythrocytes.**

Horizontal lines represent the mean (SD).
for GALK and GALT. In contrast, comparison of the 2 methods for the GALE assay revealed some discrepancies. However, the results of our assay system with patient samples and cell lines demonstrate that our method generates reliable results. It is possible that the 2-step reaction and the manual column separation of products from the reaction mixture in the radiometric assay led to more errors (9).

Recently, reports of multiplex enzyme assay used for screening of dried blood spots for lysosomal storage disorders have generated interest in the use of this assay in newborn screening (20–22). The multiplex lysosomal enzyme assay screens for 5 lysosomal enzymes, and performs the enzyme reactions separately and measures the products simultaneously by using tandem mass spectrometry. The multiplex assay described here uses a similar format to detect 3 galactose-metabolizing enzymes. This system could be adapted for use in second-tier newborn screening of dried blood spots for galactosemia, although more experiments would be needed to develop the assay for routine application.

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References