Neonatal Screening for Galactosemia by Quantitative Analysis of Hexose Monophosphates Using Tandem Mass Spectrometry: A Retrospective Study

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Background: Classic galactosemia (OMIM 230400) is an inherited disorder in the metabolism of galactose caused by deficiency of the enzyme galactose 1-phosphate uridyl transferase (EC 2.7.7.12). Galactosemia leads to accumulation of galactose and galactose 1-phosphate (gal-1-P) in blood and tissues and, if untreated, produces neonatal death or severe mental retardation, cirrhosis of the liver, and cataracts. Hence, the disorder is included in many neonatal screening programs.

Methods: We retrospectively analyzed filter-paper blood samples obtained 4–8 days postpartum for routine neonatal screening from 12 galactosemia patients and 2055 random controls. Total hexose monophosphates (HMPs) were used as a marker of gal-1-P and were assayed by negative-ion mode electrospray tandem mass spectrometry (tandem MS) with settings biased toward gal-1-P detection. The predominant precursor/product ion pair m/z 259/79 was used to quantify total HMPs by external standardization.

Results: Linear calibration curves were obtained in the range 0–8 mmol/L gal-1-P. The detection limit was 0.1 mmol/L HMP, and total CVs ranged from 13% at the detection limit to <8% at ≥1 mmol/L HMP. The method was in agreement with an alkaline phosphatase-galactose dehydrogenase method. All samples from galactosemia patients contained increased HMP concentrations (range for patients, 2.6–5.2 mmol/L; range for reference group, <0.10–0.94 mmol/L). The diagnostic sensitivity and specificity were 100% at a cutoff of 1.2 mmol/L HMP. A Duarte/classic galactosemia compound heterozygous sample could be discriminated clearly from both patient and reference samples.

Conclusion: Quantitative analysis of HMPs by tandem MS can be used in laboratory investigations of galactosemia.

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Galactosemia (OMIM 230400), a life-threatening disorder with severe symptoms in the neonatal period, is caused by deficiency of the enzyme galactose 1-phosphate uridyl transferase (GALT³; EC 2.7.7.12). In this disorder, ingestion of milk causes accumulation of galactose in the blood and urine and leads to high intracellular concentrations of galactose 1-phosphate (gal-1-P). gal-1-P is considered toxic for several tissues, especially the liver, brain, and renal tubules (1). Clinical manifestations of the disease appear shortly after ingestion of milk, predominantly as gastrointestinal, hepatic, and neurologic symptoms, including failure to thrive, vomiting, diarrhea, jaundice, and lethargy. Patients become comatose, and if treatment is not initiated early, death often occurs during the first weeks of life as a result of gram-negative sepsis or hepatic or renal failure. Treatment with a galactose-free diet causes regression of symptoms and signs within 1 or 2 weeks. However, because of the low frequency of the disease (~1:35 000 in Denmark) and suboptimal clinical awareness, diagnosis is often delayed or symptoms are
misinterpreted as sepsis or isoimmunization, leading to neonatal death or sequelae because of late intervention.

Although these considerations seem to advocate neonatal screening, this is still controversial, as illustrated by two recent comprehensive reviews with opposing conclusions (2, 3). There are no controversies over the serious implications of untreated galactosemia: neonatal death or severe mental retardation. The major issues in the discussion include the following: (a) the low frequency of the disease, which leads to an unfavorable cost–benefit ratio; (b) suboptimal screening tests; (c) clinical detection of the majority of patients; and (d) long-term complications despite early diagnosis and treatment. The long-term complications include intellectual impairment, speech disorders, cataracts, and hypergonadotropic hypogonadism in females (4–6). It has been claimed that clinical detection is adequate for this disease, and screening has been abandoned in the United Kingdom. There are, however, clear indications that patients often will be diagnosed at a stage of medical emergency that may lead to sequelae or death, and some cases will be misinterpreted as gram-negative sepsis, a frequent complication of galactosemia (3).

Several methods exist for neonatal screening for galactosemia. The simplest involves examining the urine for reducing substances (Fehling and Benedict). The commonly used tests for neonatal screening use dried-blood-spot samples (DBSSs). The method described by Paigen et al. (7) quantifies galactose and gal-1-P by a microbiological assay, but it is not suitable for automation and is sensitive to antibiotic treatment of newborns or their mothers. In addition, careful bacterial maintenance procedures are required. Measurement of GALT activity is the basis of the Beutler test (8, 9). GALT is sensitive to inactivation by heat or humidity, causing false-positive screening results, and blood transfusions can cause false-negative screening results for up to 3 months (1). On the other hand, one advantage of the Beutler test is its independence of galactose exposure and substrate accumulation, which is useful in screening samples obtained early after birth. The Paigen and Beutler assays in combination are used by many screening laboratories (2) and can detect deficiencies in GALT, galactokinase, and galactose epimerase; however, these methods have relatively high false-positive rates (3). The same disorders can also be detected by the alkaline phosphatase-galactose dehydrogenase assay (10–12), which quantifies galactose and gal-1-P.

All current methods require special reagents with limited stability, involve multistep sample preparation procedures, and are time-consuming. This report presents a novel method of screening for galactosemia. The method uses a common solvent to obtain an extract of screening samples in a single step. The extract is analyzed by a fast tandem mass spectrometry (tandem MS) protocol to determine the hexose monophosphate (HMP) content and hence, indirectly, gal-1-P, a marker of galactosemia.

**Materials and Methods**

**REAGENTS**

gal-1-P, glucose 1-phosphate, fructose 1-phosphate, and fructose 6-phosphate were purchased from Sigma. Methanol (cat. no. 322415) was obtained from Aldrich. The extraction solution and the mobile phase were a 1:1 mixture of ultra high-quality water from an in-house MilliQ system (Millipore) and acetonitrile (Aldrich). Before use, the solution was filtered through 0.45 µm nylon-66 filters (Varian Chromatography Systems) and degassed by sparging with helium.

**SUBJECTS AND BLOOD SAMPLES**

Twenty-two patients diagnosed with galactosemia in the period 1984–1998 were identified from the medical records at the Department of Clinical Genetics, Rigshospitalet University Hospital, Copenhagen, Denmark. All diagnoses were confirmed by GALT genotype analysis (Salamon MB, Christensen E, Skovby F, Brandt NJ, Schwartz M. Galactosemia in Denmark: mutations in the GALT gene, manuscript in preparation). The Danish neonatal screening program uses DBSSs collected on filter paper (type 2992; Schleicher & Schuell). Subsequent to routine analysis, the DBSSs are stored at −20 °C in a biological specimen bank (13). Residuals of the primary DBSSs were available from 12 galactosemia patients. A reference group was established by collecting 2055 random, deidentified DBSS residuals from apparently healthy infants 1 week after the samples had been analyzed by the routine neonatal screening program. Information on birth weight and postnatal age at the time of blood sampling was retained from the blood sample data forms. The effect of carbohydrate infusion was examined in 10 neonatal intensive care patients receiving intravenous infusions of 50 g/L glucose–50 g/L fructose. We evaluated this group anonymously by creating DBSSs from surplus EDTA blood drawn for routine clinical chemistry tests during short pauses of carbohydrate infusion. The stability of HMPs was studied in random DBSSs that had been stored at −20 °C for 1, 2, 5, and 10 years (160 samples for each length of storage).

**CALIBRATORS ON FILTER PAPER**

Stock solutions with different concentrations of gal-1-P were prepared in 9 g/L NaCl. Blood was collected in EDTA vials from a healthy fasting adult female. The blood was cooled to 4 °C to repress endogenous GALT activity. From each stock solution of gal-1-P, 70 µL was added to 1930 µL of prechilled blood. The mixtures were inverted gently. Blood-spot calibrators were prepared by pipetting 75 µL of the blood (with added gal-1-P) onto filter paper (type 2992; Schleicher & Schuell). The filter-paper calibrators were dried overnight at room temperature and were subsequently stored in zippered plastic bags at −20 °C.
The calibrators contained the following concentrations of added gal-1-P: 0, 0.0625, 0.125, 0.25, 0.50, 1.0, 2.0, 4.0, and 8.0 mmol/L. The stability of gal-1-P during the preparation of the calibrators was examined by assaying DBSSs created from EDTA blood supplemented with 1.5 mmol/L gal-1-P and incubated for 0, 1, 2, 3, or 24 h at 22 °C before spotting onto filter paper.

**EXTRACTION OF GAL-1-P**

To determine the optimal composition of the solvent used to extract gal-1-P from DBSSs, different mixtures of acetonitrile and water, methanol and water, and methanol and acetonitrile were tested, with the concentration of either solvent in a pair ranging from 100 to 900 mL/L in steps of 100 mL/L. The extraction solvents were used to prepare extracts of a DBSS enriched with 0.5 mmol/L gal-1-P in microtiter plates, as described below, with the following modification: to eliminate effects of solvent composition on desolvation and ion formation, all extracts were evaporated at 45 °C under a gentle stream of nitrogen gas and were redissolved in acetonitrile–water (1:1 by volume), injected into the tandem MS instrument, and counted as described.

**SAMPLE PREPARATION**

From each DBSS, one 3.2-mm diameter disc was punched into a blank well in a conical-bottomed microtiter plate. Extraction solution (150 μL) was added to each well, using an 8-channel pipette, and the plates were sealed with adhesive film (Sealing tape SI; cat. no. 236366; Nunc). Extraction was carried out by gentle rotation on an orbital shaker for 20 min, and 100-μL aliquots were transferred to fresh microtiter plates, which were sealed with adhesive film. Lint from the filter paper was pelleted by centrifugation at 1800g for 10 min to prevent blockage of the autoinjector port and tubing system.

The microtiter plates were loaded with the calibrators in triplicate in order of increasing concentration. The extent of signal carryover was <0.8%. Patient samples were measured in duplicate, and samples from the carbohydrate infusion group were measured in triplicate. Samples from the reference group were assayed as singletons. Processed microtiter plates were loaded in a Perkin-Elmer Series 200 autosampler (Perkin-Elmer Corporation). Preparation of 300 samples typically took 1–1.5 h, including punching.

**TANDEM MS**

All measurements were carried out on a API-365 tandem mass spectrometer (PE-Sciex) fitted with an electrospray source (IonSpray).

For the study of the fragmentation patterns of HMPs, the following substances were analyzed: gal-1-P, glucose 1-phosphate, fructose 1-phosphate, and fructose 6-phosphate. Each substance was dissolved in mobile phase at 20 μmol/L and was infused at a rate of 5 μL/min into the tandem MS instrument. The precursor ion was set at m/z 259, which corresponds to the molecular ion of all HMPs. Fragment ions of each compound were determined by summation of 10 product ion spectra covering the range m/z 50–300. The instrument settings used had been optimized for detection of the most intense gal-1-P fragment, at m/z 79: needle voltage, −4.9 kV; collision energy, −25 eV; nitrogen collision gas at setting 2 (manufacturer's units).

For analysis of each DBSS, 8 μL of sample extract was injected into a constant flow of mobile phase (70 μL/min) delivered by a Shimadzu LC-10 Advp pump (Shimadzu Corporation). The three product ions (m/z 79, 97, and 139) of HMPs (m/z 259) were assayed as interlaced selected-reaction monitoring functions with a dwell time of 500 ms for each transition, continuously repeated throughout each injection cycle. Instrument settings were as indicated in the fragmentation study. The flow rate gave a sample-to-sample cycle time on the order of 1.5 min.

**DATA PROCESSING**

Ion intensities were extracted from the raw data files by Neochrom Software, Ver. 1.0 (PE-Sciex) operating on a Macintosh Power PC platform. Microsoft Excel, Ver. 5.0c for Windows, was used to generate calibration curves by linear regression, using the signal obtained from the m/z 259 > 79 ion transition for standardization, except where noted. The equations describing the calibration curves of ion intensity vs gal-1-P enrichment were used to calculate the content of HMP in analyzed samples. The ion abundance ratio (IAR) of the m/z 259 > 79 transition relative to the m/z 259 > 97 transition was calculated as the ratio of the raw ion intensities in counts per second for each sample.

**STATISTICAL METHODS**

SPSS, Ver. 9.0, software was used for statistical analysis. The Mann–Whitney U-test was used to compare variables between groups. Spearman’s rank correlation test was used to investigate correlations. Linear regression analysis was used to determine confidence intervals and standard error of the estimate for the calibration curves. All tests were two-tailed and were considered statistically significant at \( P < 0.05 \) for the null hypothesis.

**ANALYTICAL PRECISION AND DETECTION LIMIT**

gal-1-P was added to aliquots of a blood sample from a healthy, fasting adult male to final concentrations of 0–7 mmol/L, and the aliquots were spotted onto filter paper and dried. Samples of each concentration were analyzed six times per assay in six independent assays carried out on separate days. CVs for HMP concentration and the IAR were determined as described previously (14). The limit of detection for HMPs was defined as 3 SD above the value obtained for the sample without added gal-1-P.
GENOTYPE ANALYSIS
The GALT genotype of a sample from the reference group with an extreme HMP concentration and IAR was determined by restriction analysis of PCR-amplified DNA extracted from the DBSS residue, as described elsewhere (Salamon MB, Christensen E, Skovby F, Brandt NJ, Schwartz M. Galactosemia in Denmark: mutations in the GALT gene, manuscript in preparation).

METHOD COMPARISON
The tandem MS method was compared with an in-house adaptation of an alkaline phosphatase-galactose dehydrogenase method (AP-GDM) (12), used by the Swedish neonatal screening program for confirmatory testing of screening samples with low GALT activity. The AP-GDM measures galactose with and without pretreatment with alkaline phosphatase to determine “total galactose” (i.e., gal-1-P plus free galactose) and free galactose, respectively, and gal-1-P is then calculated by subtraction. The highest and the lowest galactose calibrators were 4.0 and 0.1 mmol/L, respectively. Samples with added gal-1-P, some of the primary samples from galactosemia patients, and a few recall samples were assayed for gal-1-P. The means of duplicate determinations by each assay were compared by linear regression.

Results
FRAGMENTATION PATTERN OF HMPs
Tandem mass spectral analysis demonstrated that all HMPs dissociated into three predominant ionic fragments of m/z 79, 97, and 139 (Fig. 1), which is in agreement with previous findings (15). The predicted structures of the ionic fragments were \[\text{[(C}_2\text{H}_3\text{O})\text{HPO}_4]\] ^2\text{−}, \[\text{H}_2\text{PO}_4\] ^2\text{−}, and \[\text{PO}_3\] ^2\text{−}, respectively. However, there were marked differences in the relative abundance of fragments m/z 79 and 97, depending on the configuration of the HMP: The examined aldose monophosphates (AMPs; i.e., gal-1-P and glucose 1-phosphate) predominantly generate a fragment of m/z 79, whereas ketose monophosphates (KMPs; i.e., fructose 1-phosphate and fructose 6-phosphate) predominantly generate a fragment of m/z 97. As a consequence, the IAR of m/z 79 relative to m/z 97 is a measure of the chemical composition of a mixture of HMPs, with a high ratio signifying increased amounts of AMPs relative to KMPs. In other words, the IAR is an indirect measure of gal-1-P.

EXTRACTION OF GAL-1-P
The optimal extraction solvent was 300–700 mL/L acetonitrile in water, which throughout this range yielded three- and ninefold higher ion intensities than the maximal signals from samples extracted with methanol–water and methanol–acetonitrile mixtures, respectively (data not shown). Thus, acetonitrile–water (1:1 by volume) was used for extraction in all further applications.

STANDARDIZATION USING FILTER-PAPER CALIBRATORS
The ion intensities of the three predominant HMP fragments were plotted against the gal-1-P enrichment of the calibrators (Fig. 2). All three fragments produced linear calibration curves (\(r^2 = 0.994–1.000\)) at gal-1-P enrichment concentrations of 0–8 mmol/L. HMP concentrations in neonatal screening samples were 0.1 mmol/L higher, on average, when the m/z 97 fragment was used for standardization vs when the m/z 79 or 139 fragment was used. This difference was abolished in the low (<0.0625 mmol/L) HMP concentration range. The differences in calculated HMP concentrations could be attributable to a higher ratio of KMPs to AMPs or higher concentrations of interfering compound(s) in neonatal screening samples relative to the adult blood that was used to generate the filter-paper calibrators.

There was no significant difference between HMP concentrations determined by standardization using the m/z 79 or 139 fragment. Overnight incubation at room temperature of freshly drawn EDTA blood with added gal-1-P showed no evidence of signal degradation (data
not shown), as would be expected because gal-1-P was added to the extracellular compartment and was therefore not exposed to the intracellularly localized GALT. In all samples from the reference group, the patient group, and samples with added gal-1-P, the ion abundance of the m/z 79 fragment was seven- to ninefold higher on average than that of the m/z 139 fragment. In contrast, the IAR of the m/z 79 fragment relative to the m/z 97 fragment varied with sample type, and was highest in galactosemia samples, intermediate in a GALT compound heterozygous sample, and lowest in the reference samples, thus producing the characteristic hyperbolic shape in Fig. 3. In all pathological samples and a fraction of the reference samples, the intensity of m/z 79 was higher than that of m/z 97. We decided to use the ion intensity of the m/z 79 fragment for quantification of gal-1-P because it was associated with an AMP fragmentation pattern, and hence gal-1-P (Fig. 1), and gave the highest signal intensity in samples containing excess gal-1-P (gal-1-P-enriched samples and galactosemic samples).

**Analytical Precision and Detection Limit**

The detection limit for HMPs as determined by repeat analysis of a blood sample with no added gal-1-P was 0.1 mmol/L. The total CVs of the HMP analysis ranged from 13% at the detection limit to <8% at concentrations >1 mmol/L. The total CVs of the IAR analysis ranged from 14% to <4% at IAR values >1.4.

**Stability of HMPs**

Analysis of DBSSs that had been stored for various lengths of time at −20 °C revealed minor but statistically significant alterations in HMP status. Thus, the median HMP content decreased from 0.15 to 0.12 mmol/L (P = 0.001) over the course of 10.6 years, whereas the IAR increased from 0.80 to 0.84 (P = 0.01). No appropriate curve-fitting models were apparent for either association, and the changes were considered to be too minor to necessitate correction of results obtained from the analysis of archival patient samples.

**Reference Group**

A total of 2055 reference samples were available for study, 92% of which were obtained 4–8 days postpartum (median, 5 days). The median birth weight was 3550 g (range, 732–5720 g). The median concentration of HMP in the 2055 reference samples was 0.15 mmol/L (range, <0.10–0.94 mmol/L; Fig. 3). The median IAR of fragment m/z 79 relative to m/z 97 was 0.80 (range, 0.32–1.41). Neither
HMP concentration nor the IAR correlated with postnatal age at blood sampling or birth weights ≥2500 g (Fig. 4). For low-birth-weight (<2500 g) infants (n = 114), however, both HMP concentration and the IAR correlated positively with the birth weight (P < 0.01 and P < 0.05, respectively). One sample with an increased HMP concentration of 1.44 mmol/L and an increased IAR of 1.57 was shown by DNA mutational analysis to be compound heterozygous for the Duarte mutation (N314D) and a classic galactosemia mutation (Q188R) of the GALT gene, and was excluded from the reference group (Fig. 3).

**GALACTOSEMIA PATIENTS**

Characteristics of patient samples are shown in Table 1. HMP concentrations were 2.6–5.2 mmol/L, and the IAR was 1.69–1.76. There was a weak positive correlation between postnatal age at blood sampling and HMP concentration (P < 0.05), but no other correlations between the characteristics listed in Table 1.

**EFFECT OF INTRAVENOUS CARBOHYDRATE INFUSION**

The intensive care neonates received infusions of 50 g/L glucose–50 g/L fructose at a median rate of 191 mL/24 h (range, 12–312 mL/24 h). Compared with the reference group, these neonates (n = 10) had slightly but significantly repressed HMP concentrations and IARs (median HMP, 0.06 vs 0.15 mmol/L; median IAR, 0.42 vs 0.80; P < 0.001 for both comparisons). The rate of carbohydrate infusion correlated negatively with the IAR (P < 0.001), but not with HMP concentrations.

**DIAGNOSTIC ACCURACY**

In Fig. 3, the patient samples and the Duarte/classic galactosemia compound heterozygous sample are compared with the reference samples for HMP concentration and IAR. For neonatal screening, cutoffs of 0.95–2.5 mmol/L for HMP concentration and 1.42–1.68 for the IAR yielded sensitivities and specificities of 100%. If detection of Q188R/N314D compound heterozygotes is desired, 100% sensitivity and specificity can be obtained with cutoffs of 0.95–1.43 mmol/L for HMP and 1.42–1.56 for the IAR.

**METHOD COMPARISON**

Overall, there was excellent agreement between the HMP concentrations obtained by tandem MS and gal-1-P values obtained by the AP-GDM (Fig. 5). However, in 2 of 10 patient samples tested, the gal-1-P concentration determined by the AP-GDM was approximately one-half the HMP concentration determined by tandem MS. These two samples (Fig. 5, gray circles) had free-galactose concentrations (11 and 15 mmol/L, respectively) in excess of all other patient samples (median, 5.8 mmol/L; range, 0–9.5 mmol/L). The most likely explanation of the unexpectedly low gal-1-P in these cases is the saturaibility of the AP-GDM: The AP-GDM measures galactose in samples with and without pretreatment with alkaline phosphates, and the concentration difference represents gal-1-P. Measurements were done as end-point determinations, and the highest calibrator concentration was 4 mmol/L. Thus, unphosphorylated galactose concentrations approaching saturation of the AP-GDM would lead to underestimation of the gal-1-P fraction. As a consequence, the two outliers were excluded from the linear regression analysis. The methods were in perfect agreement for the remaining samples, consisting of eight patient samples and nine samples with added gal-1-P (linear regression: P < 10^-19; R^2 = 0.996; slope = 1.01; intercept = −0.04 mmol/L).

**Discussion**

We have developed a novel test that can be applied to the detection of galactosemia. The target analyte is gal-1-P. The test principle is quantitative analysis of total HMPs by tandem MS. HMPs have fragmentation patterns in tandem MS that depend on chemical structure: AMPs, such as gal-1-P, yield a m/z 79 fragment as the predominant charged fragment, whereas the predominant charged fragment for KMPs is m/z 97. For quantitative reasons and because of the specificity for AMPs, the intensity of the m/z 79 fragment was used to represent gal-1-P. The IAR, the ratio of the raw signal intensity of m/z 79 relative to m/z 97, was used as a marker of the aldose-to-ketose ratio of HMPs, and hence as an indirect measure of gal-1-P accumulation. Because no appropriate internal standard exists, we used filter-paper calibrators consisting of peripheral blood enriched with gal-1-P. In principle, this makes the method more susceptible to aberration by altered ionization efficiencies attributable to, for example, co-ionized contaminants. Although we have seen no evidence of this, stringent use of quality-control materials is imperative to control for systematic variations until internal standards are developed.
Furthermore, the IAR between fragments \( m/z \) 79 and 97 was able to distinguish clearly between the reference cohort, the compound heterozygote, and the patient cohort, with marked increases in the latter groups. In Fig. 3, it is apparent that the IAR varied greatly within the reference population, whereas the galactosemic population had tightly grouped IAR values approaching the IAR of pure gal-1-P, which can be seen in the product ion scan of gal-1-P (Fig. 1). The hyperbolic relationship between HMP concentration and the IAR seen in Fig. 3 was replicated exactly in the gal-1-P-enriched samples used to determine analytical CVs (not shown), further underscoring the relationship between the IAR and gal-1-P. A possible explanation of the curve shape is that interfering HMPs contribute to the variability of the IAR in the reference samples, which have low HMP concentrations. In galactosemia samples, the gal-1-P fragmentation pattern will dominate for quantitative reasons, and the IAR will thus be predicted to approach that of pure gal-1-P. In essence, the IAR is a pattern-specific, composite marker of galactosemia that parallels the carnitine ratios used in the diagnosis of fatty acid oxidation disorders, e.g., octanoyl carnitine relative to decanoyl carnitine for the diagnosis of medium-chain acyl-CoA dehydrogenase deficiency (16).

The IAR is independent of internal or external calibrator concentrations and corrects signal variations caused by sample inhomogeneity or variations in ionization efficiency, because both ion intensities in the IAR will be affected to nearly the same degree. We suggest a cutoff of 1.5 for the IAR, which will detect compound heterozygotes and patients with a sensitivity and specificity of 100%. It should be noted, however, that the optimal value of the IAR can differ between screening laboratories, depending on the configuration and performance of the tandem MS instrument being used.

A limitation of HMP quantification for galactosemia screening is that subjects would have to be exposed to a galactose-containing diet before screening. Thus, parental nutrition that does not contain galactose, or sampling before effective lactation can create false-negative screening results, as can blood transfusions and other measures that reduce the blood content of gal-1-P. These limitations, however, are also present with other galactosemia screening assays that depend on accumulation of disease markers, i.e., gal-1-P and galactose. On the other hand, gal-1-P analysis is insensitive to the problems of enzyme inactivation, which is the major limitation of the Beutler test and derivatives thereof.

The majority of the reference samples and all patient samples in this study were obtained between 4 and 8 days postpartum. This is routine practice in many places, but the trend is toward early hospital discharge and early screening. This raises the question of whether tandem MS will be able to detect galactosemic samples obtained at earlier postnatal ages than those used for this study. It has been shown that galactose loading of galactosemia patients produces the same pattern of intraerythrocytic...
gal-1-P accumulation regardless of age: In response to an oral load of 100 mg/kg galactose, gal-1-P concentrations in excess of 1.1 mmol/L of erythrocytes (corresponding to 0.46 mmol/L of blood) were reached within 2–4 h (17). Thus, if a cutoff of 1.2 mmol/L gal-1-P is used to detect galactosemic infants, a galactose load of 260 mg/kg would be required. The concentration of lactose (one-half of which is galactose) in colostrum increases from 40 g/L to 60 g/L from 1 day to 3 days post partum, plateauing at 70 g/L within the first week of life (18). Similarly, the median yield of colostrum in the first 24 h post partum is 37 g/24 h (range, 7–12 g/24 h). Milk production rapidly increases to a median of 408 g/24 h (range, 98–775 g/24 h) at 3 days post partum (18) and beyond throughout the first month of life. It follows that the galactose intake of newborns increases from 740 mg/24 h (median) in the first day of life to 3000–23 000 mg/24 h at 3 days post partum. The median galactose intake is thus borderline sufficient for screening 24 h post partum, whereas all intakes are quite sufficient for screening 3 days post partum. Additional factors will aid in the detection of galactosemia patients: gal-1-P is increased 10-fold in the cord blood of affected newborns, to concentrations on the order of 0.1–0.9 mmol/L (6, 19, 20). Furthermore, in affected newborns, gal-1-P will accumulate with each feed until a steady-state concentration is reached. It is thus reasonable to assume that the present method could have a role in neonatal screening in situations of early hospital discharge, although this needs to be demonstrated on samples obtained closer to the time of birth.

The galactosemia samples in this study were from patients discovered clinically and diagnosed at a mean of 18 days of age (range, 6–36 days; Table 1). Assuming a reporting delay of 2 full days from the time of sampling, neonatal screening could have advanced the time of diagnosis in 10 of 12 patients by an average of 13 days. An additional finding of this study is that galactosemia can be diagnosed retrospectively in DBSSs that have been stored frozen for at least 15 years.

Because the method quantifies total HMP content, not gal-1-P per se, it has a potential role in the diagnosis and monitoring of other disorders with affected metabolism of hexose phosphates as well as a potential for analytical interference. The instrument settings, the chosen fragment for HMP quantification, and the IAR in this study were biased toward detection of gal-1-P. In practice, signals from KMPs are suppressed by the use of the m/z 79 fragment, and an interfering KMP signal can be discriminated clearly from an AMP signal by use of the IAR, which will be low. By careful alteration of the instrument settings and selection of relevant fragments for analysis, other HMPs can be favored or detection could include multiphosphorylated hexoses. For example, hexose bisphosphates can be detected directly or as HMPs because they can be ionized to yield HMPs by postsource decay in mass spectral analysis (data not shown). Samples could also include media other than blood, e.g., urine or tissue homogenates. In a broader sense, diseases relevant to the concept of HMP analysis include hereditary fructose intolerance (OMIM 229600; increased fructose 1-phosphate), fructose 1,6-bisphosphatase deficiency (OMIM 229700; increased fructose 1,6-diphosphate), and diabetes mellitus (increased glucose 3-phosphate). In hereditary fructose intolerance, dietary fructose leads to accumulation of fructose 1-phosphate in the liver. Newborns are not usually exposed to fructose, and this in combination with a nonerythrocytic expression pattern of the enzyme makes interference in a galactosemia screening program unlikely. Similarly, gluconeogenetic enzymes such as fructose 1,6-bisphosphatase are not expressed in erythrocytes, and newborns do not have diabetes mellitus, thus eliminating interference from these diseases. HMP analysis of appropriate tissues at appropriate stages might, however, be relevant to these diseases.

The future role of this tandem MS application in laboratory investigations of galactosemia needs to be determined. The possibilities include population screening, selective screening, follow-up testing, confirmatory analysis, and monitoring of dietary therapy.

Like other screening tests for galactosemia that rely on analyte accumulation, this method requires exposure of the newborns to sufficient amounts of galactose before screening. At present, how much closer to birth than 4 days the samples can be drawn is not known, but it is unlikely that there should be any difference in comparison with the existing gal-1-P methods. The method uses a minute amount of the DBSS, which is already obtained for routine use by neonatal screening services. Sample preparation is simpler and faster than all other existing methods. Only common reagents are required. A stable analyte is quantified. Analysis and interpretation are fully automated, and the diagnostic accuracy is seemingly unsurpassed. However, the tandem mass spectrometer is operated in negative-ion mode, and rapid switching of voltages from positive to negative potentials is deleterious for power supplies. This precludes integration of the HMP procedure with methods analyzing positive ions, such as typical tandem MS screening panels covering amino acids and acylcarnitines (21, 22). The HMP procedure is also incompatible with the butyl-derivatization step used in that analysis. Thus, HMP analysis requires a separate batch procedure. The capability of tandem MS to detect multiple analytes in a single assay is heralded as its chief advantage and might seem to contradict the use of this relatively expensive technology to cover a single analyte. However, most neonatal screening laboratories will have analysis time to spare. For example, our neonatal screening center, which is typical in size, services 70 000 newborns annually, equivalent to a flow of 300 samples per work day. Analysis of this sample load for amino acids and acylcarnitines occupies one tandem MS instrument for 10 h per day, leaving time for other tandem MS applications, such as gal-1-P. In addition, tandem MS panels used for neonatal screening will almost certainly expand to cover a range of negative ions, which can possibly be integrated with the HMP procedure.

The speed and easy setup of the method as well as the lack of a requirement for diagnostic reagent sets with
limited shelf lives may make the method attractive in situations where a limited number of samples are to be analyzed on an irregular basis. This can be the case with selective screening procedures and in confirmatory testing of samples from galactosemia screening where low GALT activity has been found. In addition, untreated galactosemic infants develop hepatic insufficiency, which in many cases leads to a detectable increase in blood aromatic amino acids (23). Hence, the method lends itself to rapid follow-up analysis of routine neonatal screening samples with increased concentrations of phenylalanine and/or tyrosine.

With a detection limit for HMP of 0.1 mmol/L, the present method will not be of use in monitoring the dietary therapy of galactosemia patients. It is unlikely that adaptations to improve the analytical sensitivity will compensate for the lack of analyte specificity, i.e., the method determines total HMPs biased toward AMPs, but not gal-1-P alone, and at low gal-1-P concentrations, interference becomes prohibitive. To be useful for therapeutic monitoring, both specificity and sensitivity need to be improved, e.g., by augmenting the method with liquid chromatography.

In conclusion, we present a novel method of detecting galactosemia. We suggest that laboratories involved in neonatal screening and biochemical genetics consider this procedure as a simple, inexpensive adjunct to existing tandem MS panels.

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References