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Urine screening of five-day-old newborns: metabolic profiling of neonatal galactosuria☆

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Abstract

We determined urinary galactose and 4-hydroxyphenyllactic acid (4HPLA) in 4338 of 5-day-old newborns using a newly developed GC–MS screening method. Fifty-two infants were chemically diagnosed as having transient galactosuria based upon elevated urinary galactose levels (4.78–30.53 mg/mg creatinine, control 1.10±0.89 mg/mg creatinine). These infants did not excrete galactitol or galactonic acid into the urine, which is typical of hereditary galactosemia. Nearly 40% of the transient galactosuria was associated with immature infants (low birth weight or borne before 37 gestational weeks). Immature hepatic function is one explanation for neonatal transient galactosuria, but heterozygotes or the carriers of galactose degradation enzyme deficiencies were also suspected in some of the newborns, judging from the comparisons of urinary galactose and 4HPLA excretion between neonates and patients with galactosemia.

Keywords: Neonatal screening; Galactose; 4-Hydroxyphenyllactic acid

1. Introduction

Human urine contains many compounds that are metabolic intermediates or products of the catabolism of amino acids, fatty acids, carbohydrates, and other molecules. Many acquired and inherited metabolic disorders can be chemically diagnosed by detecting the abnormal excretion of urinary organic acids, amino acids and sugar alcohols. Recently, Shoemaker et al. [1] described an unique means of detecting urinary metabolites using gas chromatography–mass spectrometry (GC–MS). Urine is digested with urease, then organic acids, amino acids and carbohydrates are simultaneously analyzed. We modified Shoemaker's procedure and developed a more simple and rapid screening method with which to chemically diagnose inborn errors of metabolism (IEM) [2]. Using this new GC–MS method we conducted a pilot study for newborn IEM screening and discovered a relatively large number of infants with elevated levels of urinary galactose. These elevations were not detected by prior GC–MS methods because the screening consisted of organic solvent extraction, which detects only organic acids in urine.

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Galactosuria is associated with not only hereditary galactosemia, but also liver diseases. Most hepatic failure in newborns is due to liver immaturity, and transient tyrosinemia is found at high frequency in such infants. Neonatal transient tyrosinuria is caused by immature hepatic tyrosine aminotransferase or 4-hydroxyphenylpyruvic acid oxidase, so a large amount of 4-hydroxyphenyllactic acid (4HPLA) is excreted into the urine. This report describes the quantitation and comparison of urinary galactose and 4HPLA levels in infants with transient galactosuria and tyrosinuria. The present study was implemented during a screening program of 5-day-old neonates.

2. Materials and methods

2.1. Materials

Galactose, 4HPLA and *n*-heptadecanoic acid were obtained from Tokyo Kasei Kogyo (Tokyo, Japan), urease (type C-3: from Jack beans) was from Sigma (St. Louis, MO); *N,O*-bis(Trimethylsilyl)-trifluoroacetamide (BSTFA) and trimethylchlolosilane (TMCS) were from Wako Pure Chemical Industries (Osaka, Japan). Absorbent filter paper (UA-5) was purchased from Toyo Roshi (Tokyo, Japan).

2.2. Newborn urine filter paper samples

First morning urine samples from 5-day-old neonates were absorbed onto filter papers (3 cm×8 cm) and dried at room temperature. The papers were then mailed to our laboratory from several hospitals over a period of 2 years. During that period, 4338 samples were collected.

The samples were set in injector syringes, then 1 ml of distilled water was added. The samples were left undisturbed for 10 min. The urine was extracted back by centrifugation (2800 *g* for 5 min at 4°C). About 0.7 ml urine was collected, of which 0.1 ml was used for GC–MS analysis.

Urine samples were also collected from two high risk patients, one with galactosemia type I and the other with partial defect of galactosemia type III. Two other urine samples were also collected, one with transient neonatal jaundice and the other with transient hypergalactosemia. These samples were frozen at −20°C until analysis.

Urinary creatinine was enzymatically measured using a COBAS auto analyzer.

2.3. Urease digestion

Details of the analytical procedure have been published [2]. In brief, 0.1 ml of urine was digested with 30 units of urease at 37°C for 10 min. After adding of 50 nmoles of *n*-heptadecanoic acid as the internal standard, the urine was deproteinized by 1 ml ethanol. The precipitate was removed by centrifugation (10 000 *g* for 3 min), then the supernatant was concentrated under reduced pressure and evaporated to dryness under nitrogen gas. The residue was trimethylsilylated using 100 μl of BSTFA plus 10% TMCS at 80°C for 30 min, then 1 μl of the reaction mixture was analyzed by GC–MS.

2.4. GC–MS analysis and quantitation of urinary metabolites

TMS derivatives of urinary metabolites were analyzed using GC–MS (QP-5000, Shimadzu, Japan), equipped with a fused silica capillary column (J&W DB-5, 0.25 μm×0.25 mm×30 m). The column temperature was held at 125°C for 2 min then programmed to 325°C in increments of 16°C/min. Mass spectra from m/z 50 to 500 were obtained by scanning at 0.25 s intervals.

Galactose and 4HPLA were quantified by the mass chromatography. Calibration curves were prepared by comparing the ion chromatographic peak area to that of the internal standard in mass chromatography. Fragment ions m/z 204 and m/z 179 were used to identify galactose 5TMS and 4HPLA 3TMS, respectively. The ion m/z 327 of *n*-heptadecanoic acid 1TMS was used as the internal standard. Usually, the GC peak of galactose 5TMS appeared as two peaks under our analytical conditions. The second peak of galactose was higher than the first and the ion intensity ratio was 2.22±0.07. Quantitation was based on the second galactose peak. Concentrations of these analytes were determined by comparison with calibration curves and were expressed as mg/mg creatinine.

3. Results

In the chemical diagnosis of metabolic disorders, galactose and 4HPLA have been used as markers compound for galactosuria and tyrosinuria, respectively. Our new procedure allows simultaneous analysis of organic acids, amino acids and sugar alcohols. Therefore, galactosuria and tyrosinuria can be diagnosed after only one sample injection into the GC–MS.

Urinary metabolites were quantified by mass chromatography. This technique is very specific and compounds were confirmed their mass spectra. Mass chromatograms of galactose (m/z 204), 4HPLA (m/z 179) and heptadecanoic acid (internal standard, m/z 327) are shown in Fig. 1. Urinary galactose TMS derivatives appeared as two GC–MS peaks and a phenomenon that has been reported by others [1,12]. The second peak was higher than the first and they appeared at an almost constant ratio. Galactose quantitation was based on the second peak.

Fig. 2A shows a chromatogram typical of urinary metabolites from a newborn infant with galactosuria. Galactitol, galactonic acid and 4HPLA were not excreted. On the other hand, Fig. 2B shows a chromatogram obtained from a patient with galactosemia type I (8-day-old), who excreted high concentrations of galactose (53.11 mg/mg creatinine) and 4HPLA (2.56 mg/mg creatinine). Galactitol and galactonic acid were also excreted in large amounts. Fig. 3 shows chromatograms from two infants with transient tyrosinuria. The chromatogram in Fig. 3A is from an infant with no clinical abnormalities and the patient excreted increased levels of 4HPLA (6.87 mg/mg creatinine), whereas the chromatogram in Fig. 3B is from a patient (3-month-old) with jaundice and the patient excreted increased levels of 4HPLA (1.66 mg/mg creatinine), and galactose (10.98 mg/mg creatinine) was also found. These results indicate that the excretion of 4HPLA, rather than of galactose is more characteristic of an immature liver. A patient with type III galactosemia with only 34% epimerase activity, excreted high levels of galactose (12.73 mg/mg creatinine), whereas galactitol, galactonic acid and 4HPLA concentration remained normal (Fig. 4).

We defined normal urinary galactose levels in newborns by excluding the upper 1% of the values and used the mean + 3 SD (>3.77 mg/mg creatinine for galactose) as the cutoff value. The concentration of galactose was above the normal cutoff (ranging from 4.78 to 30.53 mg/mg creatinine) in 52 out of 4338 samples and 15 of those were above the mean + 10 SD (>10.03 mg/mg creatinine). Among the 52 infants with galactosuria, 21 were immature infants (born before 37 weeks of gestational age or with lower than 2500 *g* birth weight). However, galactose levels were not apparently related to either gestational age or birth weight (Fig. 5).

Sixteen infants were diagnosed with tyrosinuria because of urinary levels of 4HPLA ranging from 0.42 to 6.87 mg/mg creatinine (control 0.05±0.06 mg/mg creatinine). Among these infants, 14 were born before 37 weeks of gestation and one had a low

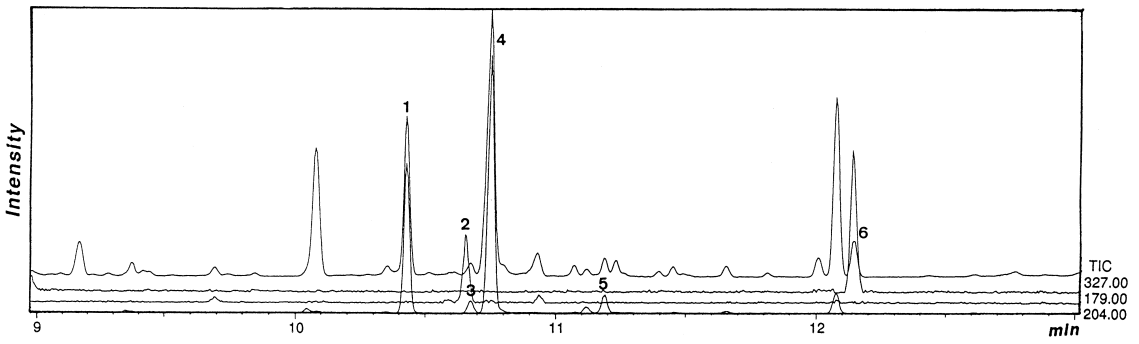

Fig. 1. Mass chromatograms of urinary metabolites. Galactose and 4HPLA were detected using the ions m/z 204 and m/z 179, respectively. The ion m/z 327 was used to detect *n*-heptadecanoic acid (internal standard). These metabolites were quantified from the ion peak area ratios. 1) galactose [1]; 2) 4-hydroxyphenyllactate; 3) glucose [1]; 4) galactose [2]; 5) glucose [2]; 6) *n*-heptadecanoate (internal standard).

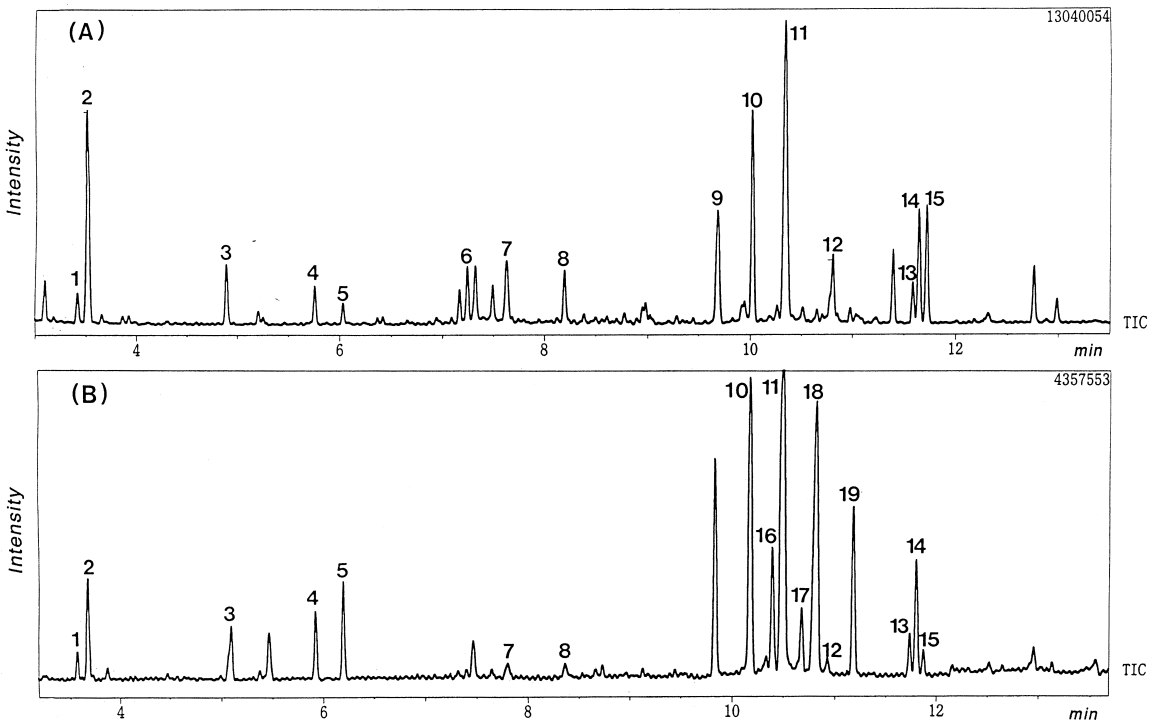

Fig. 2. Typical TIC chromatogram of urinary metabolites in transient galactosuria (A) and galactosemia type I (B). Peaks were identified by the library search program as follows: 1) alanine; 2) glycine; 3) phosphate; 4) serine; 5) threonine; 6) methionine; 7) creatinine; 8) phenylalanine; 9) citrate; 10), 11) galactose; 12) glucose; 13) urate; 14) myo-inositol; 15) *n*-heptadecanoate(I.S); 16) 4-hydroxyphenyllactate; 17) tyrosine; 18) galactitol; 19) galactonate; 20) 4-hydroxyphenylpyruvate.

birth weight. So, over 90% of infants of tyrosinuria were immature. Galactose excretion was above the mean + 3 SD but less than + 10 SD in three of them.

The relationship between the urinary excretion of galactose and 4HPLA is shown in Fig. 6. Group A consists of normal individuals with average galactose and 4HPLA concentrations of 1.09 ± 0.89 and 0.05 ± 0.06 mg/mg creatinine, respectively. Group B represents infants with transient tyrosinuria in whom the 4HPLA concentration was over 0.22 mg/mg creatinine (mean + 3 SD). Group C consists of infants with transient galactosuria in whom the concentration of galactose was over 3.77 mg/mg creatinine (mean + 3 SD).

Concentrations of galactose and 4HPLA in transient galactosuria, transient tyrosinuria and several galactosemias are shown in Table 1.

4. Discussion

Hereditary galactosemia is caused by a deficiency of the enzymes involved in galactose metabolism. There are three types of galactosemia (Fig. 7). Type I (galactose-1-phosphate uridyltransferase deficiency, McKusick 230400) manifests the most severe clinical conditions, including mental retardation, speech defects, ovarian failure and ataxia [3,4]. Patients with type I who do not undergo appropriate treatment at an early age, can develop severe liver damage. Type II is caused by a galactokinase deficiency (McKusick 230200), that most frequently appears as lens opacity. Several investigators have described lens opacity in the parents of patients with types I and II galactosemias as well as in patients with borderline levels of enzymes that degrade galactose [5–7].

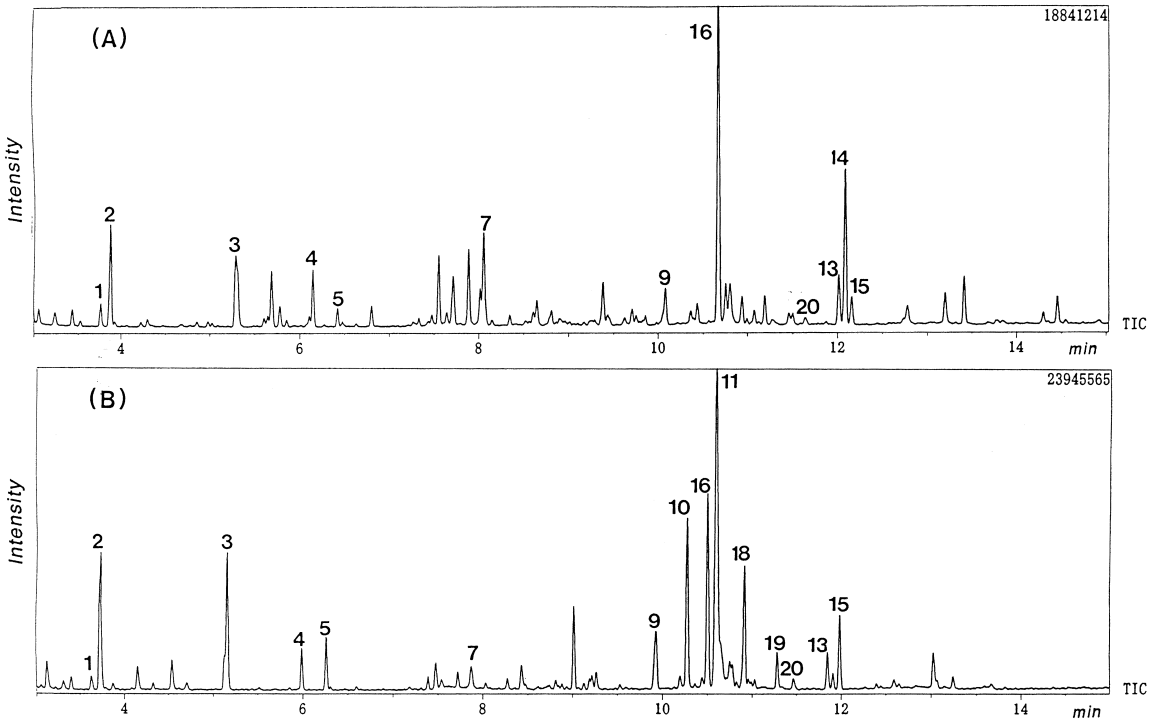

Fig. 3. TIC chromatogram typical of urinary metabolites in transient tyrosinuria (A) and jaundice (B). Peak numbers correspond to Fig. 2.

These two types of galactosemia patients excrete large amounts of galactose, galactitol and galactonic acid into the urine. Galactitol is thought to be associated with cataracts. On the other hand, patients with galactosemia type III (uridine diphosphate galactose-4-epimerase deficiency, McKusick 230350) show no clinical abnormalities and excrete low levels of galactitol. All galactosemia patients have high blood levels of galactose and galactosemia is typically screened by measuring these levels [8,9].

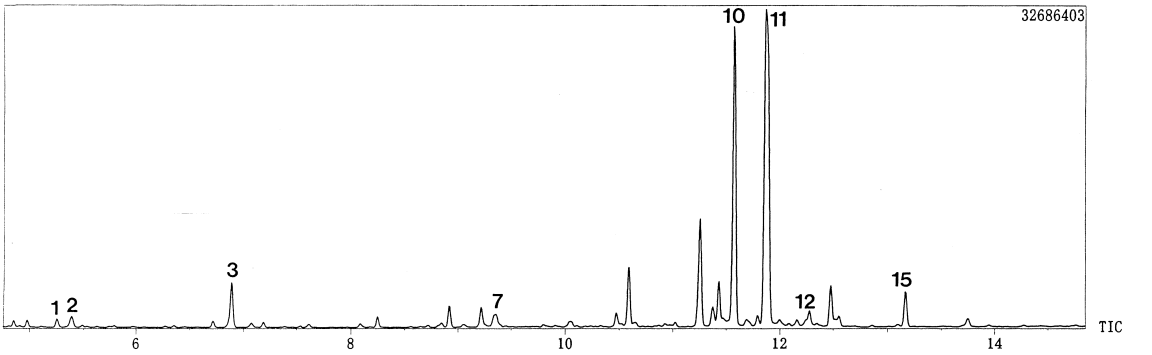

Fig. 4. TIC chromatogram of urinary metabolites in galactosemia type III. Peak numbers correspond to Fig. 2.

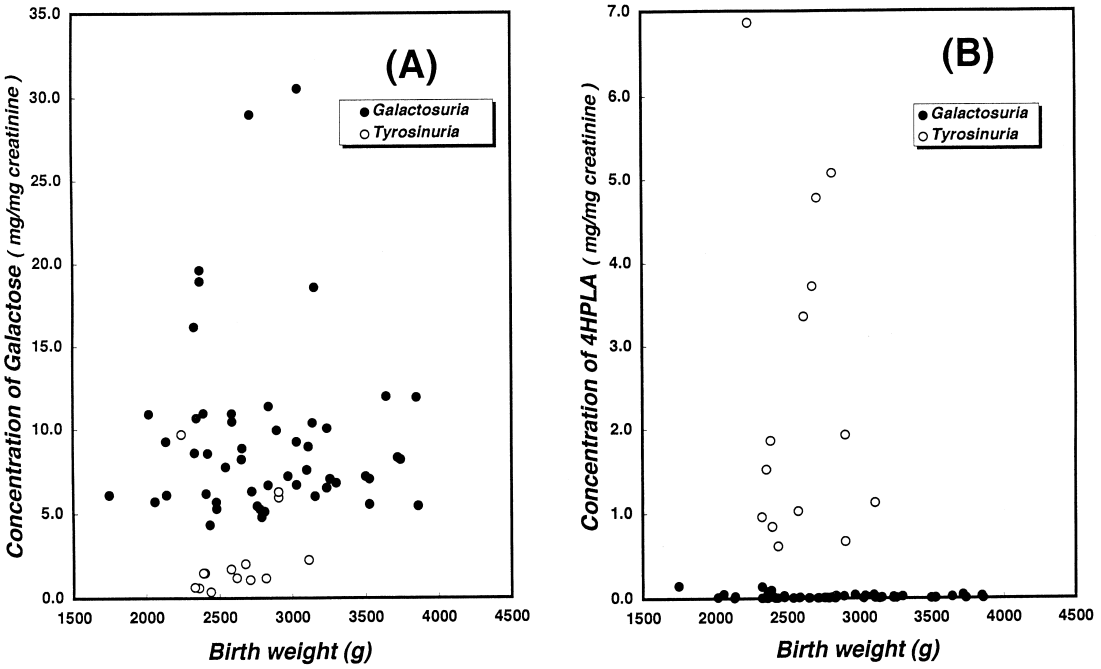

Fig. 5. Relationships between birth weight and concentrations of urinary galactose and 4HPLA in neonatal galactosuria (●) and tyrosinuria (○).

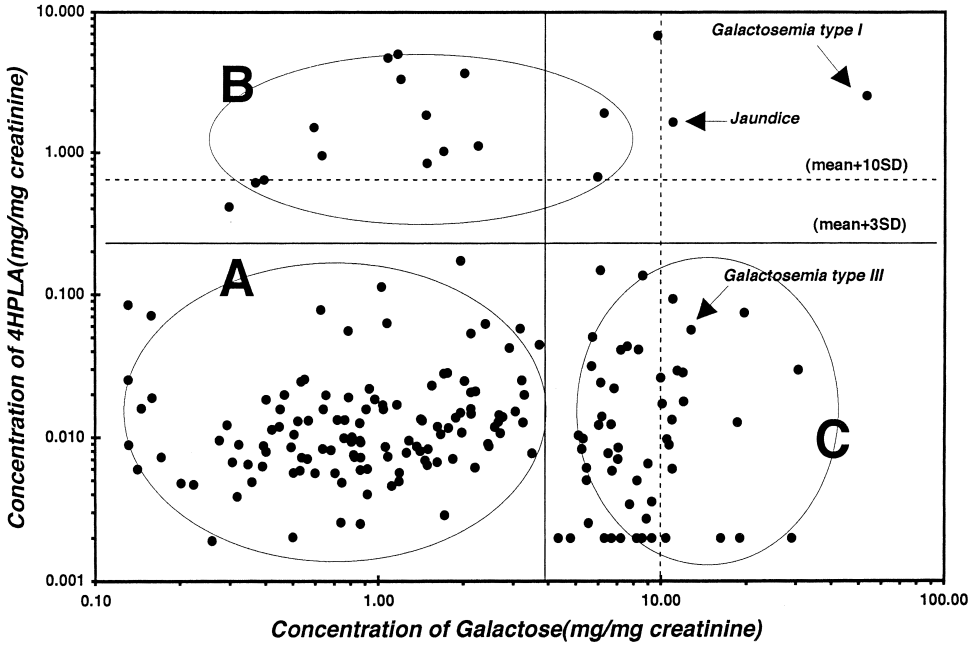

Fig. 6. Relationship between concentrations of urinary galactose and 4HPLA in 5-day-old infants. Group A, normal individual; Group B, transient tyrosinuria; Group C, transient galactosuria. Line (———) shows mean+3 SD value; (– – –) shows mean+10 SD value.

Table 1
Urinary excretions of galactose and 4-hydroxyphenyllactic acid (mg/mg creatinine)

Substance	Galactosuria (neonatal)[a] ($n=52$)	Tyrosinuria (neonatal)[a] ($n=16$)	Galactosemia Type I 8-day-old	Galactosemia Type III[b] 1-month-old	Galactosemia transient 14-day-old	Jaundice 3-month-old	Control (neonatal)[a] ($n=126$)
Galactose	4.78–30.53	0.03–9.68	53.11	12.73	22.78	10.98	1.10±0.89
4HPLA	0.01–0.15	0.42–6.87	2.56	<0.01	<0.01	1.66	0.05±0.06

[a] 5-day-old urine was analyzed.
[b] This patient has 34% residual enzyme activity.

Blood galactose-1-phosphate is another important compound for the selective diagnosis of galactosemia type I. However, this compound can not be detected by one simple analysis.

Urinary galactitol and galactonic acid are markers for the diagnosis of galactosemia. Both are excreted at high concentrations in patients with galactosemia types I and II [10,11], and lower galactitol concentrations are excreted by patients with type III. Urinary galactose and galactitol have been analyzed by GC or GC–MS [12]. However, conventional GC or GC–MS analyzes only sugar alcohols, whereas our new procedure simultaneously analyzes sugar alcohols, organic acids and amino acids in a single sample injection. Our procedure also provides additional information important for chemical diagnosis of IEM.

None of the infants with galactosuria screened by our GC–MS study developed any clinical episodes, and they did not indicate galactosemia. However, significant amounts of galactose were excreted in their urine, whereas levels of other organic acids and

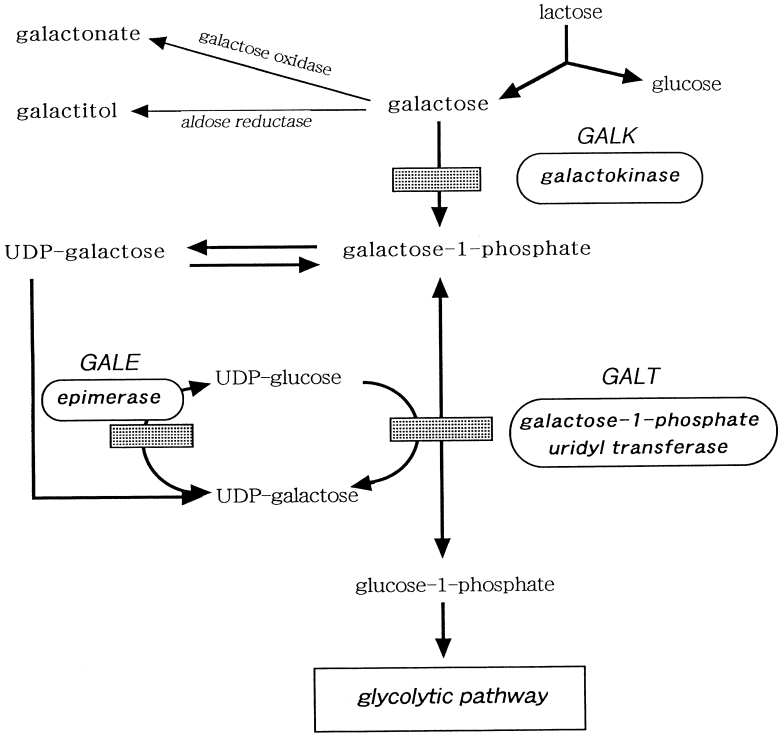

Fig. 7. Galactose metabolic pathway.

amino acids were normal (Fig. 2A). The excretion of galactose was not caused by kidney underdevelopment, because the level of excreted amino acids was normal. Large amounts of galactose are excreted in the presence of hepatic dysfunction. The most prevalent cause of hepatic failure in newborns is hepatic immaturity, and transient tyrosinemia is frequent in such infants. Hepatic tyrosine aminotransferase and 4-hydroxyphenylpyruvic acid oxidase, the key enzymes in tyrosine metabolism, mature after birth and are dependent on the degree of liver maturity. In infants with an immature hepatic system, large amounts of 4HPLA are excreted into their urine, representing a marker of transient tyrosinuria. Sixteen infants were diagnosed in our screening program as having transient tyrosinuria because excreted 4HPLA levels were very high. Over 90% of the infants we diagnosed as having transient tyrosinuria were immature.

We compared the urinary excretion of galactose and 4HPLA in newborns with transient galactosuria and with transient tyrosinuria. The excretion of 4HPLA was dependent on gestational age or birth weight. Infants born after a short gestation or with a low birth weight excreted more 4HPLA. However, galactose excretion did not correlate with gestational age or birth weight. The urinary excretion levels of 4HPLA in infants with transient tyrosinuria identified in this screening were higher than the mean + 10 SD value (0.63 mg/mg), but none of the newborns with transient galactosuria had excreted excess 4HPLA. On the other hand, high risk patients with galactosemia type I excreted extremely high levels of 4HPLA and galactose. Patients with jaundice also excrete large amounts of galactose and 4HPLA into the urine, but at lower levels than those with galactosemia type I.

A patient with a partial deficiency of epimerase (type III, with 34% residual enzyme activity) excreted a large amount of galactose but not galactitol, galactonic acid and 4HPLA, and the concentration of galactose (12.73 mg/mg creatinine) was almost equal to that excreted by infants with transient galactosuria (>10.03 mg/mg creatinine; mean + 10 SD).

A national neonatal screening program for galactosemia revealed that the diagnostically significant concentration of serum galactose is over 5 or 8 mg/dl (control is lower than 2 mg/dl) in Japan. We examined a patient with a serum galactose concentration of 5.3 mg/dl. Although galactosemia was not obvious, the urinary galactose level was 22.8 mg/mg creatinine. This patient was normalized after 1 month. This result indicates the possibility that the blood galactose levels in infants with galactosuria are borderline of cutoff point. Infants with a partial deficiency of galactose-1-phosphate uridyltransferase (GALT) were identified by Gitzelman et al. during newborn screening programs [13]. They identified 108 of these deficiencies from a total of 476 000 newborns. From a comparison of red cell galactose-1-phosphate and blood cell GALT, they suggested that 40% of the 108 were compound heterozygotes for classical galactosemia or the Duarte variant. Ichihara et al. reported that only 10% of patients with galactosemia found during the Neonatal Screening Program in Japan were homozygotes and that 17% were heterozygotes [14]. Blood galactose levels in our infants with galactosuria were within the cutoff point. However, we observed that 27% of these infants had urinary galactose levels at or above 10.03 mg/mg creatinine. These levels were of an order similar to that found in patients with a partial deficiency of the epimerase (galactosemia type III). Our screening results indicate that the immaturity of hepatic enzymes related to galactose metabolism is not the key cause of galactosuria in immature newborns because galactose was not excreted in infants with tyrosinuria. If those infants were the heterozygotes or had partial defects of galactose metabolism, they should have been detected by enzymatic screening. We conclude that urinary galactose and 4HPLA detection in neonates is a very sensitive and effective method of identifying disturbances of galactose metabolism. In addition, our screening procedure for galactosuria by GC–MS can provide valuable information in relation to enzymatic disturbances such as heterozygotes or partial deficiencies of galactose metabolic enzymes. We have also demonstrated that neonatal liver failure can be easily diagnosed using our GC–MS screening method and that this procedure will be useful for the selective and rapid diagnosis of many metabolic disorders involving hepatic failure.

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