Microassay for Estimation of Galactose and Galactose-1-Phosphate in Dried Blood Specimens

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A fluorometric assay for blood galactose and galactose-1-phosphate has been modified and improved to shorten the analysis time and to increase sensitivity above other published methods. The method may be useful as a quantitative screening or routine clinical test to detect infants suspected of having a defect of galactose metabolism. It can also be used to monitor blood galactose or galactose-1-phosphate levels in children with galactosemia who are on a lactose-free diet.

KEY WORDS: galactosemia; galactokinase; galactose-1-phosphate uridyl transferase; fluorometry.

Two main defects in galactose metabolism are known to be associated with uridyl transferase deficiency called classic galactosemia and galactokinase deficiency.

In these congenital disorders, galactose-1-phosphate (gal-1-P) or galactose (gal) accumulate in the erythrocytes and other tissues of the newborns (1, 2). Diagnosis and dietary treatment during the neonatal period can prevent hepatic damage, mental retardation, or death, and will allow relatively normal development of these children (3).

Current methods for galactosemia are mostly screening procedures measuring semi-quantitatively the concentration of gal and gal-1-P (4, 5, 5a), or detecting qualitatively the activity of erythrocyte uridyl transferase (6) in dried blood specimens.

Recently, Fujimura *et al.* (7) reported an automated microdetermination of gal and gal-1-P in dried blood spots. The method is not simple or practical, because it requires a Technicon Autoanalyzer II (Technicon Instruments Corp, Tarrytown, NY 10591) with a special fluorometer. The method of Misuma *et al.* (4) is a good technique for mass screening of galactosemia, but it is time-consuming, and not sufficiently sensitive for quantitative measurement of low levels of gal and gal-1-P in the dried blood specimens. We describe an improved and modified method, which is rapid and more sensitive for simultaneous quantitation of gal and gal-1-P in dried blood specimens.

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Materials and methods

Principle

The method measures gal and total gal and gal-1-P. The enzymatic reactions involved are shown in Figure 1. The gal-1-P is converted to gal by the addition of alkaline phosphatase. Gal is then quantitated by measuring the reduction of NAD to NADH by galactose dehydrogenase (Fig. 1).

Apparatus

Filter paper: SS No. 903; Schleicher and Schuell, Keene, NH 03431. Office puncher: to punch discs 4.8 mm (3/16 in) in diameter. Spectrofluorometer, or any fluorometer of adequate sensitivity.

Reagents

Tris buffer, 50 mmol/L. Dissolve 6 g of tris (hydroxymethyl aminomethane (base) in distilled water. Adjust the pH to 8.6 at 25°C with concentrated HCl and dilute to 1 litre.

Nicotinamide adenine dinucleotide (β'-NAD), 0.013 mol/L.

Galactose dehydrogenase (EC: 1.1.1.48). Suspension in 3.2 mol/L $(NH_4)_2SO_4$ solution. Activity: 4.7 U/mg protein (Sigma Chemical Co., St. Louis, MO 63178).

Phosphatase alkaline (EC: 3.1.3.1). Suspension in 3.2 mol/L $(NH_4)_2SO_4$. Activity: 1150 U/mg protein. (Sigma.) Make a fresh dilution (1:1) with distilled water every 3 days, and store both solutions in refrigerator.

Galactose, 25 mmol/L and galactose-1-phosphate, 25 mmol/L, stock solution (Sigma). Dissolve 45 mg D(+)-galactose and 107 mg of galactose-1-phosphate-$K_2 \cdot 5H_2O$ in 10 mL distilled water. Store in 1.0 mL aliquots at −20°C.

$$\text{GAL-1-P} + H_2O \xrightarrow[\text{PHOSPHATASE}]{\text{ALKALINE}} \text{GALACTOSE} + \text{PHOSPHATE}$$

$$\text{GALACTOSE} + NAD^+ \xrightarrow{\text{GALDH}} \text{GALACTONO-LACTONE} + NADH$$

Figure 1 — Enzymatic reactions in the assay.

Reaction mixture I

Mix 0.5 mL of tris-buffer, 0.5 mL of NAD, 8 μL (0.13 U) of galactose dehydrogenase and 8 μL (30 U) of diluted alkaline phosphatase, and keep refrigerated.

Reaction mixture II

The same as Reaction Mixture I, but the alkaline phosphatase was omitted.

Dried blood standards

Heparinized blood (out-dated) from healthy adults was used to make the standards. The hematocrit was adjusted to 55% by the removal of plasma. Gal and gal-1-P solution was added in appropriate amounts to the blood to have blood standards with concentrations of 0, 125, 250, 500, and 1000 μmol/L for each compound. The concentrations of gal and gal-1-P in these standards expressed in mg/dL whole blood were: 0, 2.2 + 3.2, 4.5 + 6.5, 9.0 + 13.0, and 18.0 + 26.0. The blood standards were spotted on filter paper with a pipet (2.5 cm diameter spot), dried at room temperature, and stored in the refrigerator. They were stable for four weeks at 0–5°C.

Blood samples

Blood specimens were collected on Schleicher and Schuell No. 903 filter paper. Two or three drops of capillary blood from a finger or heel prick were spotted on filter paper and allowed to dry at room temperature away from any source of heat.

Procedure

Two 4.8 mm (3/16 in) diameter discs were punched from each specimen and placed into two test tubes (12 × 75 mm). Twenty μL of acetone–methanol solution (1:1, v/v) was added to denature the hemoglobin, and the tubes allowed to stand for 30 min in a water bath (37°C) for complete evaporation of the solution. The tubes were placed at room temperature; 50 μL of tris-buffer was added, and the tubes were gently shaken occasionally to elute the compounds. Another tube was also set up with a plain paper (no blood) disc and 50 μL tris-buffer, as a blank and control of reagents.

After 15 min at room temperature, all the tubes were placed in a water bath at 37°C for 1–2 min. Then 50 μL of reaction mixture I was added to the first tube, and 50 μL of reaction mixture II to the second, and to the blank tubes. Exactly 20 min later, the tubes were removed from the bath, and 1.4 mL of distilled water was added to stop the reaction.

The solutions were transferred to fluorometric cuvettes, and the fluorescence was measured with excitation at 340 nm and emission at 460 nm in a spectrofluorometer that had been set at zero with the blank.

Calculations

The fluorescence of the first cuvet measures both gal and gal-1-P while the fluorescence of the second cuvet measures only gal. The difference in fluorescence (△F) measures the gal-1-P.

The concentrations of these compounds were obtained from the calibration curve.

NOTE: The gal and gal-1-P expressed in μmol/L of blood can also be calculated by the following equations using a standard blood disc, containing both compounds.

$$\text{Gal}\,(\mu\text{mol/L}) = \frac{\text{Fu}}{\text{Fst}} \times \text{Cst} \times \frac{1000}{0.007}$$

$$\text{Gal-1-P}\,(\mu\text{mol/L}) = \frac{\triangle\text{Fu}}{\text{Fst}} \times \text{Cst} \times \frac{100}{0.007}$$

where: Fu = fluorescence of unknown; △Fu = increase of fluorescence; Fst = fluorescence of standard; Cst = amount of standard (μmol); 1000 = to convert to 1000 mL of blood; ~0.007 = volume of blood (mL) in 4.8 mm (3/16 in) disc.

Results

Conditions of the assay

To obtain optimum denaturation of hemoglobin with methanol-acetone and fast evaporation of this mixture, the test tubes with the discs were placed in the water bath (37°C) for 30 min. The blood discs should be preincubated with tris-buffer for at least 15 min at room temperature to have elution of the components.

In the enzymatic reaction (Fig. 1), the activity of galactose dehydrogenase at 6.5 mU/0.1 mL of reaction mixture was found to be optimum at pH 8.6. The NADH product of the reaction gave maximum fluorescence in 16 min and then remained constant. The fluorescence of the solution after dilution with distilled water and under these conditions was stable for at least 60 min at room temperature.

Standard curve

The calibration curve was made by punching two 4.8 mm (3/16 in) diameter discs from each standard dried blood spot and assaying according to the procedure.

The readings of fluorescence after subtraction of the fluorescence of the zero standard were found to be proportional to the concentration of gal and gal-1-P and linear up to the concentration of 1000 μmol/L (Fig. 2).

Precision and sensitivity

We examined the reproducibility of the method by assaying 15 blood discs punched from different blood spots from the same specimen. The average concentration of gal was 394 ± 33 μmol/L and gal-1-P 373 ± 19 μmol/L. The coefficients of variation, 4.1% for gal and 3.9% for gal-1-P, indicated a satisfactory degree of precision for this method using dried blood specimens.

One to five 3.2 mm (1/8 in) dried blood discs were punched out from the same specimen and, after fixation of Hb and elution, were assayed for gal and gal-1-P. The fluorescence increase was found to be proportional to the number of blood discs, and was linear up to five

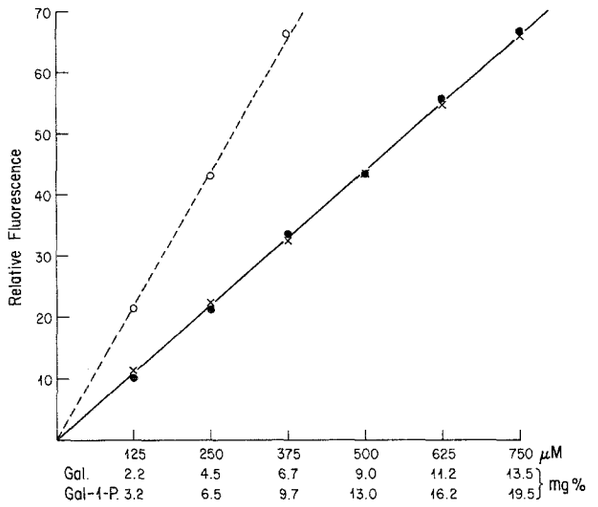

Figure 2 — Standard curve of galactose and galactose-1-phosphate in dried blood spot. Galactose (●), galactose-1-phosphate (×) and galactose plus galactose-1-phosphate together (○).

discs. When the concentrations were calculated, the mean values for gal and gal-1-P were 383 ± 16 μmol/L and 380 ± 15 μmol/L. This showed that this method is very sensitive; even a 3.2 mm blood disc will give acceptable values for gal and gal-1-P.

Stability of Gal and Gal-1-P

Dried blood specimens on filter paper were left at room temperature (21–25°C), and the concentrations of gal and gal-1-P were determined every day. There was no substantial change in the concentrations until after one week. This period of time is adequate to mail the specimens to any laboratory for testing.

When dried blood spots from the same specimens were stored in plastic bags in the refrigerator (0–5°C), the concentration of gal remained essentially the same for at least 6 weeks and the concentration of gal-1-P for more than two months.

Normal Values

In 115 dried blood specimens from apparently healthy newborn infants (3–4 days old), the mean concentrations of gal and gal-1-P were 15 ± 9 μmol/L (range 0–44 μmol/L), and 38 ± 27 μmol/L (range 0–96 μmol/L). These values were close to those reported earlier by others (8,9). These specimens had been sent by mail from a local Children's Hospital for routine lead poisoning screening.

Abnormal Specimens

Dried blood specimens from 8 patients with abnormal erythrocyte uridyl transferase activity and a patient with hepatic disorder were assayed blindly along with 10 normal specimens. The results are shown in Table 1.

In cases 4, 5, and 8 with very low activity of erythrocyte uridyl transferase, the values of gal and gal-1-P were found to be less than was expected because the infants had been placed on dietary therapy when the blood specimens were taken for analysis. In case 2, the gal was markedly increased due to the liver disorder (jaundice) but after 6 months treatment decreased close to the normal, and the erythrocyte uridyl transferase activity reached almost the normal level.

Comparison with Other Methods

Dried blood specimens from 10 newborn patients with abnormal gal and gal-1-P were assayed in parallel by our method and Paigen's method (5). Table 2 shows the results. The values for total gal and gal-1-P by our

Table 1
Galactose and Galactose-1-Phosphate Concentrations in Dried Blood Spots from Eight Infants with Abnormal Erythrocyte Uridyltransferase Activity[a]

Case No.	Patient	Activity U/g Hb	Galactose μmol/L	Gal-1-P μmol/L	Presumed Genotype
1	C.P.	9.8	33	119	D/N
2	C.J.	—	1330	123	
	C.J.	17.8 (6 mo. later)	32	15	N/N
3	C.K.	0.1	72	988	G/G
4	K.D.	3.2	44	100	D/G
5	R.C.	4.4	55	88	D/G
6	C.M.	0.4	194	1615	G/G
7	M.A.	12.0	33	69	D/G
8	O.M.	0.8	28	46	G/G
Normal Controls n = 10		19.0–23.0	0–1	4–31	N/N

D = Duarte variant gene
G = Galactosemia gene
N = Normal gene
[a]Quantitative uridyltransferase assay

TABLE 2
Comparison of Total Galactose and Galactose-1-Phosphate Concentrations by the Present and Paigen's Methods

	Present method (μmol/L)			Paigen method (μmol/L)
Case No.	Gal	Gal-1-P	Total	Gal + Gal-1-P
1	978	192	1170	1035
2	694	146	840	773
3	517	192	709	~595
4	478	596	1074	~800
5	1228	3246	4474	~3780
6	5144	1031	6175	5170
7	194	338	532	432
8	567	15	582	549
9	211	292	503	353
10	4161	177	4338	~3820
Mean			2040	1726
$r = 0.982$				

method were found to average 18.1% greater than those by the comparison method which measures the total amount of both compounds. This difference can be attributed to the fact that our method is more quantitative and more sensitive.

Five other blood specimens, three from normal infants and two from infants with gal and gal-1-P elevations, were assayed by our method with dried blood spots, and by Gitzelmann's method (1) slightly modified with liquid blood. The results of gal and gal-1-P expressed in mg per 100 mL of whole blood by both methods were found to be very close, with a correlation coefficient $r = 0.984$.

Discussion

The above results show that this fluorometric micromethod using a dried blood sample is as efficient and reproducible as are the semi-quantitative screening methods using either dried blood spots or liquid blood.

The fixation of hemoglobin with methanol–acetone mixture at 37°C, and the elution of gal and gal-1-P prior to the reaction, were more effective and required less time than in the other methods (4, 8). The enzymatic reaction under the optimum conditions described also requires less time and could be terminated in 16 min. This method can detect both gal and gal-1-P at concentrations <20 μmol/L using either 4.8 mm or 3.2 mm diameter blood discs. This concentration is lower than that of the other methods.

The mean value of gal-1-P in normal newborns determined by this method was 1.0 mg per 100 mL of whole blood, or approximately 2.0 mg per 100 mL of packed erythrocytes. This value agrees with that reported by Kirkman and Maxwell (9) (<115 μmol/L). We may assume that the normal level of gal-1-P is around 76 μmol/L of erythrocytes. The mean value of gal in these normal newborns was less than 16 μmol/L of whole blood for 86% of them, and between 16 and 44 μmol/L for the remaining 14%.

Since this method as described is sensitive and rapid, it can be used as a routine clinical or confirmatory test to detect galactosemic infants. It can also serve as a simple procedure for following children with galactosemia who are on a lactose-free diet.

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