Tohoku J. exp. Med., 1982, **137**, 289–295

Simultaneous Quantitative Estimation of Galactose-1-Phosphate and Galactose in Blood for the Diagnosis of Galactosemia

YUSHIN FUJIMURA, MASAHIKO KAWAMURA* and HIROSHI NARUSE†

*Department of Microbiology, Nagoya City Public Health Institute, Nagoya 467, *Clinic of Pediatrics, Meijo Hospital, Nagoya 460, and † National Center for Nervous, Mental and Muscular Diseases, Kodaira 187*

FUJIMURA, Y., KAWAMURA, M. and NARUSE, H. *Simultaneous Quantitative Estimation of Galactose-1-Phosphate and Galactose in Blood for the Diagnosis of Galactosemia.* Tohoku J. exp. Med., 1982, **137** (3), 289–295 —— A new microfluorometrical simultaneous assay method of galactose-1-phosphate and galactose in blood discs was devised by use of alkaline phosphatase and β-galactose dehydrogenase. Our method statistically corresponded well with the Kirkman's method. It can detect 1×10^{-10} mole of minimal concentration of galactose-1-phosphate and galactose in one blood disc paper (3 mm in diameter), and this means the sensitivity of assay of galactose-1-phosphate was 0.1 mg%. Assay range in our method was very broad (0–2 mM or 0–10 mM). The accuracy and reproducibility of galactose-1-phosphate assay were 3.4±0.1 mg%, 8.0±0.4 mg% or 15.1±0.6 mg%. Mean values of galactose-1-phosphate and galactose in blood of normal infants were 0.8 mg% and 0.3 mg%, respectively. We applied this method to mass screening of galactosemia and could accurately distinguish many positive and false positive cases detected by Paigen's and Beutler's methods. This method gave us an easy and accurate assay system for the diagnosis of uridyl transferase and galactokinase deficiencies. —— galactosemia; galactose-1-phosphate; galactose; mass screening of galactosemia

Galactose (Gal) and galactose-1-phosphate (Gal-1-P) are very important substances for the diagnosis of galactosemia. Nowadays Rommel's method (Rommel et al. 1968) for Gal and Kirkman's method (Kirkman and Maxwell 1960, 1964) for Gal-1-P are used widely and reliably. These methods, however, seem to be unfit for mass screening of galactosemia because these methods require much blood, time and labor. We described the exploitation of simple microfluorometrical assays of Gal (Fujimura et al. 1976, 1977a, b, c, 1981a, b) and UDP-galactose-4-epimerase deficiency (Fujimura et al. 1980) using one blood disc paper for mass screening. Recently we have developed it to be able to estimate simultaneously both Gal and Gal-1-P using alkaline phosphatase (AP) with β-galactose

Received for publication, June 23, 1981.

Reprint requests: Masahiko Kawamura, M.D., Clinic of Pediatrics, Meijo Hospital, 1–3–1 Sannomaru, Naka-ku, Nagoya 460.

dehydrogenase (GADH), and applied the newly devised method to the diagnosis of galactosemia types I and II (uridyl transferase and galactokinase deficiencies).

Materials and Methods

Specimens. Preparation of standard blood paper containing Gal and Gal-1-P: The hematocrit value of blood obtained before meal was adjusted to 50%. Gal and Gal-1-P in blood were determined according to the methods of Rommel et al. (1968) and Kirkman and Maxwell (1960, 1964), respectively. Gal and Gal-1-P were then separately added to blood at the final concentrations of 0.1, 0.25, 0.35, 0.50, 0.75, 1.0 and 2.0 mM. Each standard blood was dripped onto Guthrie's filter paper and dried up in air. Patient blood: Several patients with the high values detected by the Paigen's method (personal communication from Guthrie and Paigen, Ishii and Tago 1976) were selected by mass screening of about 40,000 infants.

Reagents. Gal and quinine sulfate were purchased from Katayama Chemicals (Nagoya). Gal-1-P, GADH, AP and NAD^+ were obtained from Boehringer Manheim; Guthrie's paper, from Daiichi Chemicals (Tokyo); *p*-nitrophenyl phosphate, from Sigma Chemical Co. (St. Louis); and formic acid, from Merk Co. (Darmstadt).

Denaturation of hemoglobin in blood paper by formic acid vapor. Hemoglobin in blood was denatured for the stability and accuracy of fluorometrical assay as described in detail in the previous papers (Fujimura et al. 1976, 1977c, 1981a).

Simultaneous assay of Gal and Gal-1-P. Principle: Gal-1-P was converted to Gal by AP. Gal and NAD^+ were converted to galactonolactone and NADH by GADH. Total amount of Gal derived from Gal-1-P and original Gal in blood were determined by means of fluorometry of NADH in a complete system 1. Gal alone was determined in a system omitting AP from the complete system 1 (complete system 2). The amount of Gal-1-P was thus calculated from the difference of values in the two systems. These systems were described in detail in the previous paper (Fujimura et al. 1981b). As reference fluorescence, 0.29 μM (8.4 mg in 40,000 ml of 0.01 M phospbate buffer, pH 7.4) quinine sulfate was used in assays of 0 to 2.0 mM Gal and Gal-1-P, and 1.17 μM (8.4 mg in 10,000 ml of the same buffer) quinine sulfate, in assays of 0 to 10 mM of them. Transmittances of these quinine sulfate solutions were adjusted to 100%. The calibration curves of Gal-1-P and Gal were obtained for systems 1, 2 and 3 (Fujimura et al. 1981b). The one for Gal-1-P corresponded well with the one for Gal at the same concentrations.

Effects of various buffers and enzyme concentrations on AP activities. AP activity was determined by our method (above section) and by *p*-nitrophenyl phosphate (an informational manual of Boehringer Manheim). Optimum pH was found to be 8.0, and the most suitable buffer was 0.33 to 1 M tris-HCl. AP activity was stable at concentrations of 5 μg to 5 mg/ml.

Comparison of authors' method with that of Kirkman and Maxwell in the assay of Gal-1-P. Preparation of specimens: 3 to 4 ml of packed cells were prepared from 10 ml each of normal and galactosemia (under care) blood by three times washing. The packed cells were hemolyzed by four times freezing and thawing. 50% hemolysates (equivalent to a blood sample with a hematocrit value of 50%) were used with the aim to compare our method with that of Kirkman and Maxwell (1960, 1964) at the same concentration level of Gal-1-P. The following procedures were the partial modification of the method of Kirkman and Maxwell. In order to protect Gal-1-P in blood from decomposition, first, the hemolysates of galactosemia (under care; Gal-1-P: near 0 mg%) were incubated at 37°C for 5 to 10 min to minimize endogeneous NAD^+. Then, the hemolysates were cooled at 0 to 4°C, and several known amounts of Gal-1-P were added to the hemolysates. Parts of these hemolysates containing Gal-1-P were used for the method of Kirkman and Maxwell, and the remainders were dripped onto the Guthrie's paper for our method.

Comparison of the authors' method with the Paigen's. Mean values and standard deviations of both Gal and Gal-1-P were compared using in total 94 patients who consisted

of 5 patients with the Paigen's values of over 20 mg% (P≫20 group), 9 with 20 to 12 mg% (P20~12 group), 19 with 12 to 8 mg% (P12~8 group), 25 with 8 to 6 mg% P8~6 group), 6 with 6 to 4 mg% (P6~4 group) and normal 30 with the values under 4 mg% (P<4 group).

Statistical analyses of both methods. Correlation coefficients (γ) were examined from several pairs (n: 5, 11, 18) of assay values of both methods, which were obtained by practically measuring several 50% hemolysates of galactosemia (under care; Gal-1-P, Gal: near 0 mg%) added varing amounts of Gal-1-P as shown in above section. Regression lines were expressed by the following equation; $y=bx+a$ (y: method of Kirkman and Maxwell, x: authors' method). Coefficient variations (C.V.%) were obtained using 30 each specimen of 3.4, 8.0 and 15.1 mg% standard Gal-1-P.

Results

Statistical analyses between the authors' method and that of Kirkman and Maxwell.

Gal-1-P was assayed in 50% hemolysates of galactosemia added with various known amounts of Gal-1-P using both the authors' method and that of Kirkman and Maxwell, and the results were compared statistically. The correlation coefficients (γ), a and b of regression lines were 0.999, –1.2070 and 1.1211 or 0.979, –0.3325 and 1.0231 or 0.981, 0.032 and 1.0227 when the numbers (n) were 5 or 11 or 18, respectively (Fig. 1, Table 1). The coefficient variations (C.V. %) of standards Gal-1-P were 3.5, 5.8 and 4.2% when they were 3.4, 8.0 and 15.1 mg%, respectively. The accuracy and reproducibility of the assays of Gal-1-P by the author's method were shown to be 3.4±0.1 mg%, 8.0±0.4 mg% and 15.1±0.6 mg%, respectively (Table 1). The value of 8.0±1.7 mg% (mean of errors, 3.4 mg% and 0.8 mg%) were reported for the method of Kirkman and Maxwell (1960).

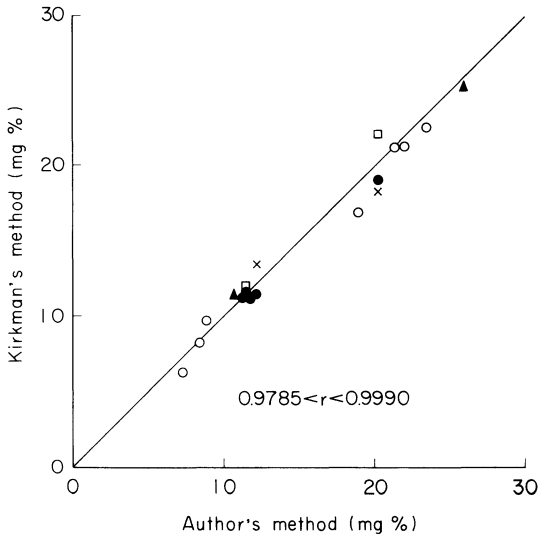

Fig. 1. Correlation between the authors' method and that of Kirkman and Maxwell. Correlation coefficients (γ) showed 0.9990, 0.9785 and 0.9990~0.9875 when numbers (n) were 5 (●), 11 (●▲□×) and 18 (○●▲□×), respectively.

TABLE 1. *The statistical values between the authors' method and that of Kirkman and Maxwell*

Article	Numbers (n)		
	5	11	18
Correlation coefficient (γ)	0.9990	0.9785	0.9809
Regression line ($y=bx+a$)			
a	−1.2070	−0.3325	0.0320
b	1.1211	1.0231	1.0227
Coefficient variation (C.V.%) of the same standard Gal-1-P Authors' method	3.5% (Gal-1-P, 3.4 mg%) 5.8% (Gal-1-P, 8.0 mg%) 4.2% (Gal-1-P, 15.1 mg%)		
Accuracy of assay of Gal-1-P by Method of Kirkman and Maxwell	8.0±1.7 mg%*		
Authors' method	3.4±0.1 mg% 8.0±0.4 mg% 15.1±0.6 mg%		
Sensitivity of assay of Gal, Gal-1-P by Authors' method	1×10^{-10} mole level		

* *J. Lab. clin. Med.* **56**, 161 (1960).

TABLE 2. *Comparison of the authors' method with the Paigen's*

Case No.	Paigen's (mg%)	Authors' (mg%)		Case No.	Paigen's (mg%)	Authors' (mg%)	
		Galactose	Galactose-1-P			Galactose	Galactose-1-P
1*	≫20	50.0	12.0	23	12～8	2.0	8.4
2*	≫20	25.0	0	24	12～8	4.0	5.6
3	≫20	22.3	3.3				
4	≫20	24.4	6.4	25	8	0.4	13.6
5	≫20	46.2	3.6	26	8	0.8	12.7
				27	8	4.5	0
6	20～12	0.9	9.3	28	8	2.6	4.2
7	12	0.4	8.1	29	8	1.2	5.8
8	12	2.0	21.4	30	8	1.0	8.4
9	12	5.8	12.6	31	8	1.0	8.4
10	12	2.2	22.9	32	8	5.8	11.2
11	12	6.2	13.4				
12	12	2.0	11.0	33	8～6	3.2	6.5
13	12	2.0	8.4	34	8～6	0	11.3
14	12	3.0	9.8	35	8～6	0	14.3
				36	8～6	5.8	2.5
15	12～8	1.0	27.1	37	8～6	2.5	6.3
16	12～8	6.4	10.1	38	8～6	0.9	9.8
17	12～8	2.9	14.7	39	8～6	1.5	12.1
18	12～8	4.6	17.9				
19	12～8	3.9	5.1	40	6	1.2	9.8
20	12～8	2.0	9.8	41	6	2.3	4.6
21	12～8	2.1	5.5	42	6	2.6	7.4
22	12～8	2.0	7.0	43	6	2.2	5.3
				44	6	0.6	1.7

* Hepatic disease (methionine 20 mg%, tyrosine 20 mg%).

TABLE 3. *Statistical comparison between the authors' and the Paigen's values*

	Concentration range of Paigen's values (mg%)					
	<4	4~6	6~8	8~12	12~20	20≪
Number of cases	30	6	25	19	9	5
Authors' values						
Mean values (mg%)						
Galactose	0.3	0.5	1.3	2.8	2.9	33.6
Galactose-1-P	0.8	4.9	7.0	9.5	12.9	5.1
Standard deviation						
Galactose	0.3	0.5	1.5	2.0	1.8	13.4
Galactose-1-P	1.0	2.2	4.3	6.0	5.6	4.5

The authors' method vs. the Paigen's

The Paigen's values were divided into six groups of P≫20, P20~12, P12~8, P8~6, P6~4 and P<4 (normal), and compared with the author's values (Table 2). Patient No. 1 showed 50 mg% Gal and 12 mg% Gal-1-P, who suffered from hepatic disease and showed 20 mg% of both methionine and tyrosine in blood. Patient No. 2 was suspected of galactokinase deficiency because of high Gal values (Gal, 25 mg%; Gal-1-P, 0 mg%), but this patient was found to be also suffering from hepatic disease with 20 mg% of both methionine and tyrosine in blood. Generally the Paigen's values show total amounts of Gal including Gal, Gal-1-P, UDP-Gal and lactose. Table 2 shows that most of P12~8 group were normal in assays of Gal by the author's. Table 3 indicates the means ($\bar{X}$) and the standard deviation (S.D.) of Gal and Gal-1-P among six groups. In P≫20 group, all patients had large amounts of Gal and small amounts of Gal-1-P ($\bar{X}$ of Gal and Gal-1-P: 33.6 mg%, 5.1 mg%; S.D.s of Gal and Gal-1-P: 13.4, 4.5). On the other hand, in patients in the P<20 groups including P20~12, P12~8 and P8~6, amounts of Gal were very small ($\bar{X}$: 2.9, 2.8, 1.3 mg%; S.D.s: all under 2), while Gal-1-P values were very large ($\bar{X}$: 12.9, 9.5, 7.0 mg%; S.D.s: 5.6, 6.0, 4.3). These patients were normal in the Beutler's test. Finally in P6~4 and P<4 groups, concentrations of both Gal-1-P and Gal were very small, and maximal values of Gal-1-P and Gal for thirty infants in P<4 group were 2.5 and 1.7 mg% (Gal-1-P, Gal of mean values: 0.8 mg%, 0.3 mg%). These values are in normal range and nearly corresponded with normal values in the reference of Kirkman and Maxwell (1960).

DISCUSSION

Mass screening of infants for galactosemia has been carried out mainly by the methods of Beutler (Beutler and Baluda 1966) and Paigen (personal communication from Paigen and Guthrie, Ishii and Tago 1976). The former involved many false positive cases resulting from enzyme inactivation. The latter also could not avoid various false positive cases, because the method determines total amounts of Gal consisting of Gal, Gal-1-P, UDP-Gal and lactose.

We succeeded in exploiting simultaneous micro-fluorometric assay of both Gal

and Gal-1-P, and applied this new method to the practical mass screening of galactosemia. This method determines both Gal and Gal-1-P by the combined use of AP and GADH. Moreover, the sensitivity and reproducibility of this micro-fluorometric method are remarkably increased through introduction of the denaturation procedure of blood disc papers which we have developed (Fujimura et al. 1976, 1977c, 1981a, b). This method can detect both Gal and Gal-1-P of the order of 1×10^{-10} mole using only one blood disc paper each of 3 mm diameter. In the assay of Gal-1-P, the method of Kirkman and Maxwell has been used in many laboratories owing to the high sensitivity (8.0 ± 1.7 mg%). Our method having the accuracy of 3.4 ± 0.1 mg%, 8.0 ± 0.4 mg% and 15.1 ± 0.6 mg% seems to be better than that of Kirkman and Maxwell. Moreover, it can be applied to the assay of blood concentrations of 0 to 2 mM, 0 to 10 mM or higher concentrations over 10 mM. On the other hand, the method of Kirkman and Maxwell is hardly applicable to Gal-1-P over 40 mg% (1.54 mM) and needs a troublesome dilution of sample so as to maintain the concentration below 15 mg% for an accurate assay. Our method has many merits which the method of Kirkman and Maxwell does not have as discussed previously.

Mean value of Gal-1-P in normal infants determined by our method was 0.8 mg per 100 ml blood or 1.6 mg per 100 ml packed cells. This value agrees with that reported by Kirkman and Maxwell (1960, under 3 mg per 100 ml packed cells). We may assume that the normal level of Gal-1-P is lower than 5 to 6 mg per 100 ml packed cells. In addition, we have obtained interesting results as shown in Table 2. All patients with the Paigen's values of over 20 mg% showed very high concentrations of Gal and low concentrations of Gal-1-P, and those with the values under 20 mg% showed reverse values. The Beutler's test showed normalcy for all patients. In cases of high values of Gal-1-P, the activity of some enzyme systems such as UDP-Gal uridyl transferase and UDP-Gal-4-epimerase which exist normally in blood may have been transiently inhibited for some unknown reason. We think that many patients with such high values of Gal-1-P might be heterozygotes of galactosemia or transient-galactosemia. In fact, the patients No. 1 and No. 2 in Table 2 were later diagnosed as hepatic disease because of high secretion of tyrosine and methionine, and because of normalcy in the Beutler's test, although these patients were initially supposed to have each transferase deficiency and kinase deficiency because of high accumulation of Gal-1-P or Gal.

There remains a possibility that our method may also detect other galactose derivatives. AP acts on many organic phosphates, but in our method its action is restricted to only Gal derivatives such as Gal-1-P and Gal-6-P by the combined use of AP and GADH. Still Gal-6-P could be the target of our method, although the content of Gal-6-P in blood is thought to be less than that of Gal-1-P.

At any rate, we consider that this new method using the combination of AP and GADH can be used adequately for mass screening of transferase- and galactokinase-deficiencies. Recently we have succeeded in automated analysis by this new method with satisfatory results (Fujimura et al. 1981b).

References

1) Beutler, E. & Baluda, M.C. (1966) A simple spot screening test for galactosemia. *J. Lab. clin. Med.*, **68**, 137–141.
2) Fujimura, Y., Yonezawa, S. & Sato, T. (1976) Mass screening for inborn errors of metabolism. (VII) Improvement of new simple micro quantitative method of galactose determination and its practical approach for mass screening. *Annual Paper of Nagoya City Public Health Institute*, **23**, 15–21. (Japanese)
3) Fujimura, Y., Yonezawa, S., Sato, T. & Kawamura, M. (1977a) Mass screening for inborn errors of metabolism. (II) Simple micro semi-quantitative method of galactose determination in blood and urine by fluorescence. *Jap. J. Pediat.*, **30**, 1521–1526. (Japanese)
4) Fujimura, Y., Yonezawa, S., Sato, T. & Kawamura, M. (1977b) Mass screening for inborn errors of metabolism. (III) Simple micro semi-quantitative method of galactose determination in blood and urine by fluorescence, and its practical application to patients. *Jap. J. Pediat.*, **30**, 1527–1528. (Japanese)
5) Fujimura, Y., Kawamura, M. & Naruse, H. (1977c) A new method of galactose estimation in blood for mass screening of galactosemia. In: *Papers on Galactosemia*, published by Boehringer-Mannheim-Yamanouchi Co. (Japanese)
6) Fujimura, Y., Kawamura, M. & Naruse, H. (1980) A new mass screening method of detecting UDP-galactose-4-epimerase deficiency. *Tohoku J. exp. Med.*, **131**, 15–22.
7) Fujimura, Y., Kawamura, M. & Naruse, H. (1981a) A new method of blood galactose estimation for mass screening of galactosemia. *Tohoku J. exp. Med.*, **133**, 371–378.
8) Fujimura, Y., Ishii, S., Kawamura, M. & Naruse, H. (1981b) Microdetermination of galactose and galactose-1-phosphate in dried blood spots. *Analyt. Biochem.*, **117**, 187–195.
9) Ishii, S. & Tago, K. (1976) Mass screening for galactosemia using Paigen's method. *Jap. J. clin. Path.*, **24**, 1022–1024. (Japanese)
10) Kirkman, H.N. & Maxwell, E.S. (1960) Enzymatic estimation of erythrocytic galactose-1-phosph ate. *J. Lab. clin. Med.*, **56**, 161–166.
11) Kirkman, H.N. & Maxwell, E.S. (1964) Enzymatic estimation of galactose-1-phosphate in red blood cells. In: *Laboratory Manual of Pediatric Micro- and Ultramicro-Biochemical Techniques*. 3rd. ed., edited by D. O'Brien & F.A. Ibbott, A Hoeber Medical Book, Harper & Row Publishers, Denver, pp. 120–122.
12) Rommel, K., Bernt, E., Schmitz, F. & Grimmel. K. (1968) Enzymatische Galactosebestimmung im Blut und oraler Galactose-toleranztest. *Klin. Wschr.*, **46**, 936–940.